LUMA ALAÍS PEDROSO

ETHANOL AND VOLATILES FROM CASTOR BEAN CAKE ON PLANT-PARASITIC NEMATODES CONTROL

LAVRAS – MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia área de concentração em Fitopatologia, para a obtenção do título de Doutora.

Prof. Dr. Vicente Paulo Campos
Orientador

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APROVADA em 04 de março de 2020.

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RESUMO

A maioria dos fitonematoides tem como alvo preferencial o sistema radicular das plantas, impedindo a translocação e a absorção de nutrientes, causando preocupação para os produtores. Por isso, é sempre importante a busca por novas estratégias de controle, uma vez que as estratégias atuais não satisfazem o agronegócio. Alguns compostos orgânicos voláteis (COVs) emitidos por plantas são tóxicos a fitonematoides e podem resultar no desenvolvimento de novos nematicidas. Nesse trabalho foram avaliados os compostos γ-decalactona, escatol, fenol e 4-metilfenol, emitidos por torta de mamona quando incorporada ao solo, contra *Meloidogyne incognita*, bem como o composto etanol no controle de *Heterodera glycines*. Exceto o fenol, todos os compostos oriundos da torta de mamona reduziram a eclosão de juvenis de segundo estádio (J2) de *M. incognita*. Houve redução da infectividade e da reprodução quando os J2 estiveram em contato com a CL_{50} dos compostos e foram inoculados em plantas de tomate, bem como quando os compostos foram aplicados no momento da inoculação com J2 na concentração de 500 mg L^{-1}. Fenol e 4-metilfenol causaram alta porcentagem de mortalidade de J2 e a fumigação do substrato contendo ovos de *M. incognita* com os compostos na concentração de 1000 mg L^{-1} substrato reduziu o número de galhas e ovos. Ao avaliar o uso do etanol contra J2 de *H. glycines* em solução e como fumigante o composto foi mais eficaz por contato direto do que apenas por fumigação em baixa concentração e a eclosão de J2 foi altamente reduzida por contato direto. A fumigação do solo infestado por *H. glycines* com etanol reduziu a infectividade e a reprodução do nematoide. Etanol na concentração de 48% reduziu significativamente o conteúdo lipídico do J2, além de alterar internamente o corpo do nematoide. A infectividade e a reprodução dos J2 também foram reduzidas quando os J2 estiveram em contato direto com etanol a 6%. Portanto, os compostos γ-decalactona, escatol, fenol e 4-metilfenol agem como nematicidas no ciclo de vida de *M. incognita*. Já, o etanol é tóxico para *H. glycines* em baixas concentrações, afetando seu comportamento patogênico, além de reduzir o conteúdo lipídico.

Palavras-chave: COVs. Nematoide do cisto. Nematoide das galhas. *Heterodera glycines*. *Meloidogyne incognita*.

ABSTRACT

The most plant-parasitc nematodes target the root system of plants preferentially and prevent translocation and absorption of nutrients, causing concern to producers. Therefore, the search for new control strategies is always important, because current strategies do not satisfy agribusiness. Some volatile organic compounds (VOCs) emitted by plants are toxic to plant-parasitic nematodes and may result in the development of new nematicides. In this work, we evaluated the compounds γ-decalactone, skatole, phenol and 4-methylphenol emitted by castor bean cake when incorporated into the soil against *Meloidogyne incognita*, as well as the ethanol compound on *Heterodera glycines* control. With the exception of phenol, all the compounds emitted by castor bean cake reduced hatching of *M. incognita* second-stage juveniles (J2). Infectivity and reproduction decreased when J2 were in contact with the LC_{50} of the compounds followed by inoculation into soil with tomato plants, as well as when the compounds were applied at 500mg L^{-1} at the time of inoculation with J2. Phenol and 4-methylphenol caused a high percentage of J2 mortality and the fumigation of substrate containing *M. incognita* eggs with the compounds at 1000mg (L substrate)$^{-1}$, reduced the number of galls and eggs. When evaluating ethanol use against *H. glycines* J2 in solution and as well as a fumigant, the compound at low concentration was more effective by direct dipping than by fumigation, and J2 hatching was highly reduced by direct dipping. Fumigation of *H. glycines* infested soil with ethanol reduced nematode infectivity and reproduction. Ethanol at 48% concentration significantly reduced the J2 lipid content, in addition to internally altering the nematode body. Infectivity and reproduction of J2 were also reduced when the nematodes were in direct dipping with 6% ethanol. Therefore, the compounds γ-decalactone, skatole, phenol and 4-methylphenol act as nematicides in the *M. incognita* life cycle. Moreover, ethanol is toxic to *H. glycines* at low concentrations and affects its pathogenic behavior rather than simply reducing the lipids.

Key words: VOCs. Cyst nematode. Root-knot nematode. *Heterodera glycines*. *Meloidogyne incognita*.

SUMÁRIO

PRIMEIRA PARTE.....8

1 INTRODUÇÃO.....8

2 REFERENCIAL TEÓRICO.....9

2.1 Compostos orgânicos voláteis.....9

2.1.1 Dinâmica dos COVs no solo.....9

2.1.2 COVs como agentes quimiotáticos em fitonematoides.....10

2.1.3 Modo de ação dos COVs em fitonematoides.....10

2.2 Plantas e seus subprodutos na produção de compostos voláteis tóxicos a fitonematoides.....11

2.3 Microrganismos produtores de compostos voláteis tóxicos a fitonematoides.....15

3 CONIDERAÇÕES FINAIS.....17

REFERÊNCIAS.....18

SEGUNDA PARTE – ARTIGOS.....23

ARTIGO 1 – Activity against *Meloidogyne incognita* of volatile compounds produced during amendment of soil with castor bean cake.....23

ARTIGO 2 – Nematicidal activity of ethanol solutions on soybean cyst nematode *Heterodera glycines*.....42

1 INTRODUÇÃO

Os nematoides fitoparasitas causam perdas consideráveis na produção de muitas culturas e estão entre os patógenos mais difíceis de controlar. Um dos principais meios de controle é o uso de nematicidas comerciais baseados em produtos químicos (TERRA et al., 2018). No entanto, o cenário do controle químico de fitonematoides está em constante mudança por causa das regulamentações cada vez mais rigorosas devido ao seu impacto prejudicial ao meio ambiente e a toxicidade ao homem (ZASADA et al., 2010).

O desenvolvimento de novos nematicidas não é uma tarefa fácil. O custo com a pesquisa e o registro de novos produtos químicos com potencial nematicida são obstáculos a serem superados (CHITWOOD, 2002). O modo de parasitismo dos fitonematoides também contribui para esse cenário, já que a maioria passa todo o seu ciclo de vida no solo ou dentro das raízes das plantas, ambientes que promovem a degradação e adsorção de moléculas nematicidas. Além disso, a cutícula do nematoide somada a outras estruturas superficiais de seu corpo são impermeáveis a muitos compostos orgânicos (CHITWOOD, 2002). Por isso, é importante buscar novas alternativas para o controle de fitonematoides.

As plantas sintetizam uma grande diversidade de compostos orgânicos voláteis (COVs) que promovem sua comunicação e interação com o ambiente (DUDAREVA et al., 2006), atuando na atração de polinizadores e dispersores, ou na proteção contra patógenos, parasitas e herbívoros (MARI et al., 2016). Ao longo dos anos mais de 1700 COVs foram identificados a partir de 90 famílias de diferentes plantas (KNUDSEN et al., 2006), podendo elevar este número à medida que mais espécies são analisadas. No entanto, fungos e bactérias presentes no solo e na serapilheira também podem produzir uma ampla variedade de COVs. A produção de COVs por microrganismos do solo tem uma importante participação na química atmosférica, nos processos do solo e nas interações bióticas, podendo regular interações microbianas inibindo ou estimulando o crescimento e a atividade de fungos e bactérias do solo (LEFF; FIERER, 2008).

Os COVs são geralmente lipofílicos com baixo peso molecular e alta pressão de vapor à temperatura ambiente. As propriedades físicas destes compostos lhes permitem atravessar livremente as membranas celulares e serem liberados no ambiente quando não existem barreiras à difusão (PICHERSKY et al., 2006).

A identificação dos COVs envolvidos nas interações específicas de plantas e microrganismos com o ambiente pode resultar no desenvolvimento de novos nematicidas,

talvez, ainda com maior garantia de segurança ao meio ambiente e aos seres humanos do que os nematicidas químicos sintéticos tradicionais (CHITWOOD, 2002).

A atividade nematicida dos COVs é interessante principalmente por causa da baixa CL_{50} evidenciada em vários trabalhos desenvolvidos (CHOI et al., 2007; SEO et al, 2014; AISSANI et al., 2015; PEDROSO et al., 2019). Portanto, esta revisão trata dos progressos obtidos no uso de compostos voláteis como uma medida de controle alternativo de fitonematoides, sejam eles emitidos por plantas e seus subprodutos (resíduos industriais) ou por microrganismos.

2 REFERENCIAL TEÓRICO

2.1 Compostos orgânicos voláteis

2.1.1 Dinâmica dos COVs no solo

Os COVs são compostos oriundos do metabolismo secundário de plantas ou de microrganismos e agem como infoquímicos, promovendo a comunicação e a interação entre os seres vivos, além de serem promissores como produtos químicos devido à capacidade de serem eficazes contra uma ampla gama de fitopatógenos (WEATLEY, 2002; CAMPOS et al., 2010). Sua atividade na rizosfera pode ser expandida ao longo do solo, seja por difusão aquosa ou atmosférica, bem como pelo movimento da água no perfil do solo, que também pode transportar esses compostos voláteis pelo sistema (WHEATLEY, 2002; TERRA et al., 2017). De acordo com Barros et al. (2014) esses compostos voláteis podem ser degradados ou retidos pela água, sendo que a retenção desses compostos pela água está relacionada à sua solubilidade. Ruiz et al. (1998) verificaram uma maior adsorção pelo solo de compostos polares comparado aos compostos aromáticos e alifáticos, dentre os quais, a argila apresenta maior capacidade de adsorção deles do que a areia. A adsorção de COVs também é afetada pela água, que diminui a retenção dos compostos em maior extensão para os aromáticos e alifáticos comparados aos compostos polares. Sendo assim, a retenção de compostos voláteis tóxicos a fitonematoides na água e a adsorção deles no solo desempenham um papel importante na sua mobilidade.

2.1.2 COVs como agentes quimiotáticos em fitonematoides

Há uma grande diversidade e complexidade de COVs que atuam contra fitonematoides e é crescente o número de pesquisas sobre a quimiotaxia desses compostos em nematoides (NIU et al., 2010; CHENG et al., 2017; ZHAI et al., 2018), em que é avaliada a orientação ou movimento do nematoide em relação aos compostos. Cheng et al. (2017) identificaram 11 COVs emitidos por um isolado da bactéria *Paenibacillus polymyxa* e verificaram que cinco deles atuavam como agentes quimiotáticos. Enquanto acetona, 2-decanol e acetona furfural atuaram como atrativos para *Meloidogyne incognita*, 2-undecanona atuou como repelente e o ácido 4-acetilbenzóico mostrou-se como atrativo em baixa concentração e como repelente em alta concentração. Entre esses compostos, acetona furfural e 2-decanol atraíam *M. incognita* e depois o matavam por contato ou fumigação. Zhai et al. (2018) também verificaram que sete COVs emitidos por um isolado da bactéria *Pseudomonas putida* atuavam como repelentes contra *M. incognita* e cinco deles (dissulfeto de dimetila, 2-nonanona, 2-octanona, acetato de (Z)-hexen-1-ol e 2-undecanona) apresentavam atividade nematicida.

2.1.3 Modo de ação dos COVs em fitonematoides

Os COVs matam os nematoides por mecanismos que podem afetar o sistema nervoso, a cutícula, o intestino, a faringe ou outros tecidos (CHENG et al., 2017). Entretanto, poucos estudos demonstram o efeito tóxico de compostos voláteis nos fitonematoides em relação ao sítio de ação. Cheng et al. (2017) expuseram juvenis de segundo estádio (J2) de *M. incognita* a 250 mg L^{-1} de 2-nonanona ou 100 mg L^{-1} de 2-decanona por 48h e observaram em microscópio óptico que os compostos destruíram a integridade do intestino e da faringe dos nematoides.

Zhao et al. (2017) avaliaram o efeito do volátil (*E*)-2-hexenal em *Bursaphelenchus xylophilus* e verificaram forte atividade nematicida por meio da fumigação, além de redução da capacidade de dispersão do nematoide no pinheiro. Além disso, os autores avaliaram seu modo de ação e verificaram que o composto perturbou os processos bioquímicos e fisiológicos normais desta espécie evidenciados por diversos fatores: 1) ligeiro aumento no teor de proteína, bem como a atividade de algumas enzimas desintoxicantes como a esterase, podendo ser um dos principais mecanismos nematicidas do composto; 2) o conteúdo de carboidratos diminuiu após 12 e 24 horas, o que pode ser devido ao aumento no metabolismo de carboidratos para resistir ao estresse, e aumentou depois de 48h, pela possível redução do

vigor do nematoide e atividade metabólica enfraquecida; 3) o conteúdo lipídico aumentou após o tratamento com (*E*)-2-hexenal, indicando que os lipídios podem contribuir para a defesa contra estresses externos; 4) o teor de trealose, açúcar que atua como fonte de energia e proteção contra estresses ambientais, aumentou; 5) a atividade da trealase aumentou inicialmente, o que pode ajudar a resistir ao estresse do (*E*)-2-hexenal convertendo a trealose em energia metabólica, e; 6) as atividades de lipases e proteases (enzimas digestivas) diminuíram após 48h, indicando interrupção da homeostase do nematoide com o tratamento do volátil.

Os isotiocianatos são compostos tóxicos para uma variedade de pragas e patógenos de solo, incluindo fitonematoides (LAZZERI et al., 2004; ZASADA; FERRIS, 2003) e podem, portanto, ser usados como fumigantes naturais chamados biofumigantes (NTALLI; CABONI, 2017). Tais compostos são biocidas gerais cuja atividade resulta da interação com proteínas de maneira inespecífica e irreversível. Desse modo, esses compostos inativam enzimas para formar produtos estáveis, devido à interação com proteínas e aminoácidos, reagindo com grupos sulfidrila, pontes dissulfeto e aminas (BROWN; MORRA, 1997).

O etanol é um composto que tem sido usado como desinfetante na concentração de 70% em soluções aquosas (MISHRA, 1993). Além disso, também tem sido utilizado na redução de fungos de pós-colheita no tratamento de frutas (ROMANAZZI et al., 2012). Em fungos fitopatogênicos seu principal modo de ação se dá pelos danos causados na membrana celular (DAO; DANTIGNY, 2011). Já em fitonematoides, o modo de ação ainda não está bem definido, mas sabe-se que o composto atravessa a cutícula dos nematoides e as camadas de proteção do ovo, causando morte ou danos severos aos nematoides e deixando os embriões desprotegidos (SILVA et al., 2017).

2.2 Plantas e seus subprodutos na produção de compostos voláteis tóxicos a fitonematoides

Do ponto de vista ecológico, as principais funções dos voláteis de plantas são defendê-las contra herbívoros e patógenos ou fornecer uma vantagem reprodutiva ao atrair polinizadores e dispersores de sementes (MARI et al., 2016). Entretanto, COVs de plantas também são conhecidos por terem atividades antimicrobianas, inseticidas e nematicidas, sejam os voláteis oriundos da parte aérea (BARROS et al., 2014), da raiz (CRESPO et al., 2012), de óleos essenciais (JARDIM et al., 2018) ou de subprodutos industriais de plantas (PEDROSO et al., 2019). Os voláteis de plantas podem ser divididos em três classes

principais: terpenoides, fenilpropanoides/benzenoides e derivados de ácidos graxos. Os terpenoides constituem a maior classe de metabólitos secundários de plantas com muitos representantes voláteis, seguido pelos derivados de ácidos graxos que incluem compostos como metil jasmonato, (Z)-3-hexenol (álcool) e (*E*)-2-hexenal (aldeído), abundantes no reino vegetal. Já os fenilpropanoides e benzenoides são voláteis estruturalmente diversos e abundantes envolvidos na reprodução e defesa de plantas, que podem originar uma variedade de aldeídos, álcoois e ésteres. Vários aminoácidos servem também como precursores para uma grande variedade de voláteis de plantas, incluindo aldeídos, álcoois, ésteres, ácidos e moléculas voláteis contendo nitrogênio e enxofre (DUDAREVA et al., 2006).

A biofumigação é uma técnica utilizada em campo definida como um processo que ocorre quando compostos voláteis com propriedades pesticidas são liberados durante a decomposição do material vegetal (KIRKEGAARD et al., 1993), podendo ser otimizado com o uso de cobertura plástica. Entre as várias plantas utilizadas na biofumigação, as espécies de brássicas têm demonstrado um efeito supressor atribuído a vários compostos biologicamente ativos, incluindo os isotiocianatos (NAGLA et al., 2015), que são liberados após hidrólise enzimática de glucosinolatos pela enzima mirosinase (BONES; ROSSITER, 2006). Barros et al. (2014) observaram o efeito nematicida de COVs emitidos por macerado de *Brassica juncea* (mostarda) contra J2 de *M. incognita*, bem como a redução do número de galhas e ovos. Análise feita por cromatografia gasosa acoplada a espectrometria de massas (GC-MS) identificou a presença de 32 compostos emitidos pelo macerado de mostarda, entre eles álcoois e compostos contendo enxofre, principalmente os isotiocianatos. Lazzeri et al. (2004) testaram diversos isotiocianatos contra *M. incognita* e verificaram que os compostos isotiocianato de 2-feniletilo, de benzilo, de 4-metiltiobutilo e de prop-2-enilo apresentaram alta atividade nematicida nos testes *in vitro*, com CL_{50} de 11, 15, 21 e 34 µmol/L, respectivamente. Zasada e Ferris (2003) avaliaram os valores de CL_{50} de sete isotiocianatos comercialmente disponíveis (isotiocianatos de alila, de benzila, de butila, de etila, de fenila, de 2-feniletila e de 4-metil-sulfonila) contra *Tylenchulus semipenetrans* e *M. javanica* em um ensaio em areia, aproximando-se da situação do solo. Os sete isotiocianatos testados variaram em suas toxicidades para as duas espécies de nematoides, com os valores de CL_{50} variando de 10 a 250 µmol L^{-1} para *T. semipenetrans* e de 40 a 260 µmol L^{-1} para *M. javanica*.

Embora os isotiocianatos desempenhem um papel importante no antagonismo a fitonematoides, outros compostos contendo enxofre também podem estar envolvidos. Vários compostos voláteis que contém enxofre têm sido encontrados em emissões de espécies de *Brassica* com atividade nematicida, entre eles o dissulfeto de dimetila (GU et al., 2007;

HUANG et al., 2010; CABRERA et al., 2014; BARROS et al., 2014), que tem sido utilizado no controle de fitonematoides (ZASADA et al., 2010). Os compostos trissulfeto de dialila e dissulfeto de dialila, encontrados em óleo essencial de alho, também apresentaram atividade nematicida contra juvenis de *Bursaphelenchus xylophilus* com valores de CL_{50} 2,79 e 37,06 μL L^{-1}, respectivamente (PARK et al., 2005).

Outras classes de compostos que também têm sido frequentemente encontradas em emissões voláteis de plantas atuando como nematicidas: álcoois, ésteres e cetonas. Barros et al. (2014) encontraram álcoois e ésteres como os principais grupos de compostos voláteis em macerados de nim (*Azadirachta indica*), apresentando atividade nematicida contra *M. incognita*. Silva et al. (2017) avaliaram a eficácia do composto etanol no controle de *M. incognita* e verificaram seu efeito nematicida tanto *in vitro* quanto em plantas de alface mantidas em casa de vegetação. Pedroso et al. (2019) encontraram o composto y-decalactona, um éster, em emissões voláteis de torta de mamona incorporada ao solo e exibiu baixa CL_{50}, 7,96 mg L^{-1}, contra *M. incognita*. Seo et al. (2014) avaliaram a atividade nematicida de 28 compostos ésteres contra *B. xylophilus*, e cinco mostraram forte atividade nematicida a 1 mg mL^{-1}. Os valores de CL_{50} para tiglato de 3-metilbutilo, isobutil-2-metilbutanoato, 3-metilbutil-2-metilbutanoato, 3-metil-2-butenil-2-metilbutanoato e pentil-2-metilbutanoato foram 21,8; 28,4; 32,6; 40,2 e 48,0 mg L^{-1}, respectivamente. Álcoois, ésteres e cetonas também foram identificados por Estupiñan-López et al. (2017), bem como por Pedroso et al. (2019), emitidos por torta de algodão e de mamona, respectivamente, incorporadas ao solo, apresentando atividade nematicida contra *M. incognita*. Ntalli et al. (2011) encontraram cetonas como componentes principais emitidos por *Ruta chalepensis* exibindo atividade nematicida contra *M. incognita* e *M. javanica*. O composto que se destacou foi 2-undecanona, que apresentou CL_{50} de 22,5 e 20,6 mg L^{-1} para *M. incognita* e *M. javanica*, respectivamente.

Os óleos essenciais de plantas contêm misturas complexas de compostos voláteis destacando-se os terpenos, que desempenham um papel importante na defesa contra insetos e patógenos, incluindo fitonematoides (D'ADDABBO et al., 2014). Os terpenos são formados pela ligação de diferentes números de unidades de isopreno, que tem como base cinco carbonos, e podem ou não conter oxigênio (terpenoides e terpenos) (NTALLI; CABONI, 2012). Oka et al. (2000) avaliaram a eficácia nematicida de diversos óleos essenciais e seus principais componentes e verificaram que os terpenos carvacrol, t-anetol, timol e (+)-carvona imobilizaram J2 e inibiram a eclosão de *M. javanica* a >125 μL L^{-1}. A atividade nematicida de 22 monoterpenoides foi avaliada *in vitro* e em experimentos em vasos por Echeverrigaray et al. (2010). Vinte monoterpenoides reduziram, significativamente, a eclosão e 11 reduziram a

mobilidade de *M. incognita* a 250 mg L^{-1}, com destaque para borneol, carveol, citral, geraniol e α-terpineol. Ntalli et al. (2010) estudaram a atividade nematicida de 13 terpenos constituintes de óleos essenciais contra *M. incognita*. Os valores de CL_{50} após imersão dos J2 em soluções de L-carvona, pulegona, (*E*)-anetol, eugenol, carvacrol, geraniol, timol e terpinen-4-ol por 24 horas foram 115, 150, 170, 256, 264, 237, 390 e 392 mg L^{-1}, respectivamente.

Outra classe de compostos que também podem estar presentes nos óleos essenciais é a dos aldeídos, que também podem apresentar ação tóxica a fitonematoides. Oito compostos presentes em óleos essenciais foram testados contra *M. javanica* por Oka (2001), entre eles alguns aldeídos. Entre os derivados de anisol testados, o *p*-anisaldeído apresentou a maior atividade nematicida nas soluções e no solo e os valores de CL_{50} do (*E*)-cinamaldeído para imobilização do J2 e inibição da eclosão *in vitro* foram baixos (15 e 11,3 µL L^{-1}). Em vasos de 3L os compostos (*E*)-cinamaldeído, 2-furaldeído, benzaldeído e carvacrol a 100 mg kg^{-1} reduziram a formação de galhas. Além disso, o tratamento do solo com o (*E*)-cinamaldeído dissolvido em etanol (50 mL m^{-2}) reduziu o número de galhas. Ntalli et al. (2016) constataram que o aldeído (*E*, *E*)-2,4-decadienal apresentou atividade nematicida contra J2 de *M. incognita*, *M. javanica* e *M. arenaria*, com CL_{50}/4dias de 8,06, 9,04 e 12,90 mg L^{-1}, respectivamente, e inibiu a eclosão de J2 de *M. incognita* a 10 mg L^{-1}. Houve redução de mais de 50% na eclosão quando as massas de ovos foram tratadas com (*E*)-2-decenal a 1 mg L^{-1}. Ambos os compostos, (*E*)-2-decenal e (*E*, *E*)-2,4-decadienal, interromperam o ciclo de vida de *M. incognita* em vasos (CL_{50} = 77,46 e 114,47 mg kg^{-1}). Jardim et al. (2018) também encontraram um aldeído presente no óleo essencial de *Cinnamomum cassia*, o (*E*)-cinamaldeído, que causou a imobilidade e mortalidade de J2 de *M. incognita*, além de reduzir a eclosão, a partir de 52 µg mL^{-1}. O número de galhas e ovos de *M. incognita* em plantas de soja também foi reduzido quando o composto foi aplicado no momento da inoculação com J2 a partir de 104 mg L^{-1}, ou atuando como fumigante em substrato infestado com ovos na concentração de 1,0 mL L^{-1}.

Compostos da classe dos fenóis emitidos por plantas ou seus subprodutos também têm demonstrado atividade nematicida, como os compostos 4-metilfenol e fenol, que apresentaram valores de CL_{50} de 70,81 e 18,68 mg L^{-1} contra *M. incognita* (PEDROSO et al., 2019). O composto 4-metilfenol também se destacou no trabalho de Yang et al. (2015), com melhor eficiência no controle de *M. javanica*.

2.3 Microrganismos produtores de compostos voláteis tóxicos a fitonematoides

Compostos voláteis são comumente produzidos por bactérias e fungos e emitidos para o meio ambiente (CAMPOS et al., 2010). As informações de um banco de dados estabelecido (mVOC 2.0 Database) mostra que mais de 1800 COVs microbianos já foram identificados. No entanto, como esses compostos podem possuir outras fontes no meio ambiente, além do metabolismo microbiano, dificilmente um determinado composto pode ser considerado exclusivo de apenas uma espécie microbiana (KORPI et al., 2009).

Os fungos se movem por meio do seu crescimento, e para se proteger dos microrganismos rivais ou para manipular o ambiente ao redor eles utilizam a emissão de COVs, e alguns desses compostos são eficazes contra fitonematoides (WERNER et al., 2016). O processo no qual a produção de COVs fúngicos inibe o crescimento de patógenos em sementes, plantas e solos agrícolas é chamado de micofumigação (ALPHA et al., 2015). Yang et al. (2012) investigaram os COVs liberados por *Trichoderma* sp. e observaram que o composto 6-pentil-2H-piran-2-ona apresentou atividade nematicida >85% para *Panagrellus redivivus*, *Caenorhabditis elegans* e *Bursaphelenchus xylophilus* em 48 h a 200 mg L^{-1}. Dois isolados de *Fusarium oxysporum* e um isolado de *F. solani* oriundos de massas de ovos de *M. exigua* também produziram COVs que resultaram em alta mortalidade (88-96%) de J2 *M. incognita* (FREIRE et al., 2012). Terra et al. (2018) testaram oito COVs mais abundantes produzidos por um isolado de *F. oxysporum* contra *M. incognita* e os compostos acetato de 2-metilbutilo, acetato de 3-metilbutilo, acetato de etilo e acetato de 2-metilpropilo causaram alta mortalidade de J2. Tais compostos apresentaram CL_{50} de 236, 198, 213 e 218 mg L^{-1}, respectivamente. No trabalho de Riga et al. (2008) o fungo *Muscodor albus* mostrou COVs com propriedades nematostáticas e nematicidas contra quatro espécies de fitonematoides: *M. chitwoodi*, *M. hapla*, *Paratrichodorus allius* e *Pratylenchus penetrans*.

Misturas sintéticas de COVs antimicrobianos têm sido usadas no controle de fitonematoides. Uma mistura sintética constituída pelos principais voláteis produzidos por *M. albus*, incluindo cetonas, ésteres e álcoois, proporcionou melhor controle do que a formulação da cultura de *M. albus* para *M. incognita*, tanto *in vitro* quanto em tomateiro. *In vitro*, apenas 5 mL de mistura sintética por mL de água foram necessários para causar 100% de mortalidade de *M. incognita* após 24 horas de aplicação (GRIMME; ZIDACK, 2007). Fialho et al. (2012) avaliaram o potencial nematicida de uma mistura sintética de COVs constituída de álcoois e ésteres, previamente identificados sendo produzidos pela levedura *Saccharomyces cerevisiae*. A fumigação de substratos contendo J2 de *M. javanica* com essa mistura de COVs apresentou

efeito nematicida de 100% nas concentrações de 66,6 e 133,3 μL g^{-1}. Liarzi et al. (2016) avaliaram a ação dos COVs do fungo *Daldinia cf. concentrica* contra *M. javanica* e verificaram uma redução de 67% na viabilidade de J2. A aplicação de uma mistura sintética dos COVs do fungo também reduziu a viabilidade (99%) e a eclosão (87%) de J2, bem como o número de galhas e ovos quando aplicada no solo inoculado com *M. javanica*.

Muitas bactérias podem produzir COVs abundantes que desempenham um papel significativo na comunicação entre organismos atuando como infoquímicos (WHEATLEY, 2002) e possuem diversas bioatividades, como ação contra fitopatógenos (ZHENG et al., 2013; POPOVA et al., 2014; XU et al., 2015). Gu et al. (2007) identificaram 22 isolados de bactérias do solo que apresentaram 100% de atividade nematicida contra *P. redivivus* e sete isolados contra *B. xylophilus*. Os compostos voláteis detectados em isolados representativos incluíam álcoois, aldeídos, cetonas, alcenos e éteres. Nove compostos (fenol, 2-octanol, benzaldeído, benzeneacetaldeído, decanal, 2-nonanona, 2-undecanona, ciclohexeno e dissulfeto de dimetila) apresentaram 100% de atividade nematicida para ambos os nematoides. Huang et al. (2010) avaliaram o efeito dos COVs de *Bacillus megaterium* contra *M. incognita*. A atividade nematicida dos COVs foi de 100% e a eclosão de J2 foi inibida completamente. Após análise por GC-MS os principais voláteis nematicidas produzidos pela bactéria foram benzenacetaldeído, 2- nonanona, decanal, 2-undecanona e dissulfeto de dimetila, os quais foram ativos contra J2 e ovos na dose de 0,5 mmol. No trabalho de Sheoran et al. (2015) os compostos voláteis da bactéria *Pseudomonas putida*, isolada de raízes sadias de pimenta-do-reino, inibiram uma ampla gama de fitopatógenos, entre eles o nematoide *Radopholus similis* (92%).

Em outro estudo, os voláteis de cinco estirpes bacterianas isoladas a partir de esterco de vaca identificadas como *Pseudochrobactrum saccharolyticum*, *Wautersiella falsenii*, *Proteus hauseri*, *Arthrobacter nicotianae* e *Achromobacter xylosoxidans*, apresentaram atividade nematicida > 91% contra *C. elegans* e *M. incognita*. Os compostos S-metil tiobutirato, 3,3-dimetilacrilato de etilo, 1-metoxi-4-metilbenzeno e isovalerato de butilo emitidos pelas bactérias também exibiram atividade nematicida (XU et al., 2015). Onze COVs foram isolados de *Paenibacillus polymyxa* no trabalho de Cheng et al. (2017) incluindo cetonas e álcoois, dos quais oito deles tinham atividade nematicida de contato, seis apresentaram atividade fumigante e cinco agiam como agentes quimiotáticos para *M. incognita*. Acetona furfural e 2-decanol atraiam *M. incognita* e depois o matavam por contato ou fumigação e 2-nonanona e 2-decanona destruíram o intestino e a faringe dos nematoides. Compostos voláteis emitidos por *Pseudomonas putida* foram avaliados contra *M. incognita*

por Zhai et al. (2018) e dos sete COVs encontrados, todos atuaram como repelentes a J2 e inibiram a eclosão de J2, tanto pelo contato direto quanto pela fumigação. Destes, cinco tiveram forte atividade nematicida contra J2 de *M. incognita* por contato direto e apenas 2-undecanona atuou como fumigante.

3 CONSIDERAÇÕES FINAIS

Estamos enfrentando novos desafios no controle de fitonematoides devido à crescente preocupação com os riscos potenciais dos produtos químicos para com a saúde humana e ambiental e consequente retirada de nematicidas químicos do mercado. Os estudos sobre a produção de COVs por microrganismos ou plantas precisam ser melhor avaliados quanto ao seu potencial no controle de fitonematoides *in vitro* e *in vivo*, bem como a translocação desses compostos no solo e seu modo de ação no fitonematoide. Deste modo, a identificação de COVs produzidos por plantas ou microrganismos envolvidos nas interações específicas com o ambiente pode resultar no desenvolvimento de novos nematicidas comerciais. Portanto, o progresso da pesquisa com COVs envolvendo fitopatógenos é necessário para o benefício da ciência e da produção agrícola.

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1 - Activity against *Meloidogyne incognita* of volatile compounds produced during amendment of soil with castor bean cake

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Elaborado de acordo com as normas do periódico Nematology.

Summary – Incorporation of castor bean cake into the soil results in the emission of four main compounds: skatole, γ -decalactone, 4-methylphenol and phenol. The toxic effects of these compounds on the life cycle of *Meloidogyne incognita* were studied here. Only phenol did not reduce hatching of *M. incognita* second-stage juveniles (J2) at any of the concentrations tested. The other compounds reduced J2 hatching by 42.1 to 59.3% at the highest concentrations. When J2 were immersed in Tween 80® solutions of each compound, at their respective LC_{50} values and inoculated into soil with tomato plants, reductions in the numbers of galls (48.5-69.3%) and eggs (42.8-62.5%) were observed in comparison with the control; this result was similar to that of carbofuran. The highest reductions in the numbers of galls (21.8-88.5%) and eggs (49.6-98.9%) occurred with all compounds when the highest concentration (500 mg l^{-1}) was applied to the substrate at the moment of J2 inoculation. Volatile compounds of γ -decalactone and skatole failed to cause J2 immobility or mortality. However, phenol caused 95.8% and 4-methylphenol caused 100% mortality. With fumigation, the highest concentration (1000 mg (l substrate)$^{-1}$) of any tested compound reduced gall numbers at a similar rate to the nematicide dazomet (98.7-100%) and reduced egg numbers (87.1-99.7%). The volatile compounds skatole, γ -decalactone, 4-methylphenol and phenol have a nematicidal effect on the life cycle of *M. incognita* and alter its pathogenic behaviour on the plant.

Keywords – γ -decalactone, 4-methylphenol, nematicide, nematode control, phenol, plant-parasitic nematodes, root-knot nematode, skatole, VOCs.

Agriculture continually faces major challenges in improving and ensuring food production for a growing and urbanised population. The limitations to these goals include climate change, degradation and loss of soil fertility, the depletion of water resources and the incidence of plant diseases and pests. Due to these constraints, professionals in the field of nematology face difficult challenges in improving plant health and productivity in fields infested with plant-parasitic nematodes (Sikora *et al.*, 2018).

Plant-parasitic nematodes cause considerable damage and loss of many crops (Luc *et al.*, 2005). Although difficult to quantify, a reduction of approximately 11% in world food production due to plant-parasitic nematodes has been estimated (McCarter, 2009). The most important group of plant-parasitic nematodes, based on scientific and economic importance worldwide, are those of the genus *Meloidogyne*, also known as root-knot nematodes (Jones *et al.*, 2013). The species *M. incognita* is the most widely distributed and perhaps the most harmful plant-parasitic nematode in the world (Trudgill & Blok, 2001).

Although there are many plant-parasitic nematode control strategies, there is always a need for new control methods. Volatile organic compounds (VOCs) with nematicidal effects present a promising means of control. Such compounds have a high vapor pressure under normal conditions, causing them to occur mostly as volatiles in the atmosphere (Yuan *et al.*, 2009). The production of VOCs in the soil probably has an important influence on atmospheric chemistry, soil chemical processes and biotic interactions (Leff & Fierer, 2008).

Several studies with VOCs have been conducted in recent years with the aim of developing new products for control of plant-parasitic nematodes. VOCs are emitted by fungi (Freire *et al.*, 2012; Pimenta *et al.*, 2016; Terra *et al.*, 2017), bacteria (Gu *et al.*, 2007; Huang *et al.*, 2010; Zhai *et al.*, 2018) and plants and their by-products (Barros *et al.*, 2014a, b; Estupiñan-Lopez *et al.*, 2017; Nasiou & Giannakou, 2018; Barros *et al.*, 2019; Silva *et al.*, 2019).

Some volatile emissions have been shown to be effective in controlling plant-parasitic nematodes by *in vitro* testing against second-stage juveniles (J2) and under glasshouse conditions, reducing nematode infectivity and reproduction (Salem & Mahdy, 2015; Yang *et al.*, 2015). However, only a few studies have reported on the nematicidal activity of the constituents of these emissions, which are obtained through gas chromatography coupled with mass spectrometry (GC-MS) (Gu *et al.*, 2007; Huang *et al.*, 2010; Cheng *et al.*, 2017; Zhai *et al.*, 2018). These compounds may be useful for the nematicide-producing industry.

The role of volatile compounds from processed oilseed residues, such as castor bean cake, has been investigated for controlling plant-parasitic nematodes (Lopes *et al.*, 2009;

Dinardo-Miranda & Fracasso, 2010; Santos *et al.*, 2013). Four volatile compounds – skatole, γ -decalactone, 4-methylphenol and phenol (Fig. S1) – emitted by the mixture of castor bean cake and soil showed toxicity to J2 when in direct contact (Pedroso *et al.*, 2019). However, the toxicity of the volatiles of these compounds has not been studied, nor has its effect on the different phases of the *M. incognita* life cycle. The compounds skatole, phenol and 4 methylphenol have been demonstrated to be insect repellents and the latter two, antibacterial and antifungal disinfectants (Degenkolb *et al.*, 2011). The toxicity of γ -decalactone has been demonstrated for plantpathogenic fungi and bacteria (Chambers *et al.*, 2013).

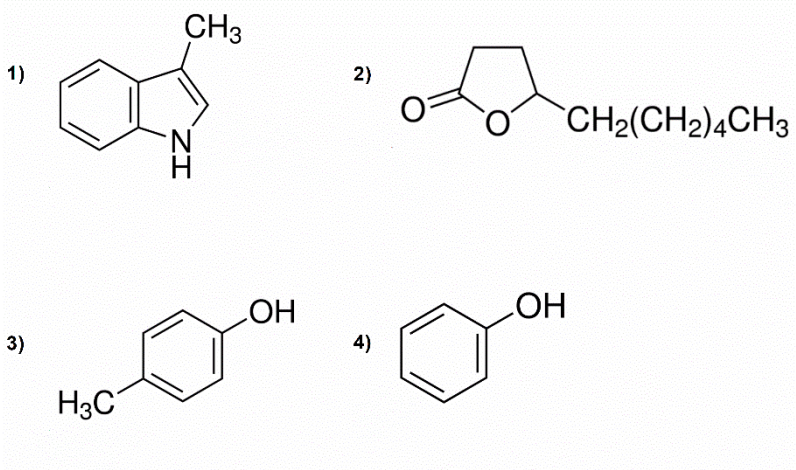

Fig. S1. Structures of the compounds selected for the study. 1: skatole; 2: γ -decalactone; 3: 4-methylphenol; 4: phenol.

The present study investigated the efficacy of skatole, γ -decalactone, 4-methylphenol and phenol at different concentrations on the hatching, infectivity, and reproduction of *M. incognita*. Also, the effects of the volatiles of the compounds on J2 immobility and mortality, and on infectivity and reproduction of *M. incognita* eggs, were investigated.

Materials and methods

MELOIDOGYNE INCOGNITA AND CHEMICALS

A pure *M. incognita* population was multiplied on tomato plants (*Solanum lycopersicum* 'Santa Clara') and maintained in a glasshouse at an average temperature of 28°C with a photoperiod of 12 h:12 h (light:dark) for approximately 2 months. The eggs were extracted from the roots with galls using a 0.5% sodium hypochlorite (NaOCl) solution

(Hussey & Barker, 1973). The extracted eggs were used in the tests or placed in a Baermann (1917) funnel to obtain the J2. Only the J2 that hatched after 24 h were used in the tests.

The compounds skatole (3-methylindole; 98%), γ -decalactone (98%), 4-methylphenol (98%), phenol (99%) and carbofuran (98%) (used as a positive control in direct contact testing) were purchased from Sigma- Aldrich and prepared at the desired concentrations using 1% Tween-80 as a diluent. Basamid® nematicide (BASF AG) with the a.s. dazomet (3,5-dimethyl-1,3,5- thiadiazinane-2-thione; 98%) (used as a positive control in volatile testing) was purchased from a local agriculture supplier.

IN VITRO HATCHING OF *M. INCOGNITA* J2 FROM EGGS EXPOSED TO THE COMPOUNDS

An aqueous suspension (0.5 ml) containing approximately 500 eggs of *M. incognita* was placed in a microtube of 1.7 ml volume. Then, 0.5 ml of each of the compounds (skatole, γ -decalactone, 4-methylphenol or phenol) dissolved in 1% Tween® 80 aqueous solution was added to the microtube. The concentrations of the compounds in the microtubes were adjusted to reach 100, 300 and 500 mg l^{-1} (chosen based on preliminary tests). However, the values of each compound at different concentrations corresponded to the following values (according to their molecular weights): γ -decalactone: 0.6, 1.8 and 2.9 mmol l^{-1}; skatole: 0.8, 2.3 and 3.8 mmol l^{-1}; phenol: 1.1, 3.2 and 5.3 mmol l^{-1}; and 4-methylphenol: 0.9, 2.8 and 4.6 mmol l^{-1}. Sterilised water and 1% Tween-80 were used as negative controls and carbofuran at 400 mg l^{-1} as a positive control. The microtubes were sealed with PVC plastic film and kept at 28°C in an incubator for 7 days. Finally, the total numbers of hatched J2 was counted.

MELOIDOGYNE INCOGNITA INFECTIVITY AND REPRODUCTION IN TOMATO PLANTS AFTER J2 EXPOSURE TO THE COMPOUNDS AT THE LC_{50}

An aqueous suspension (0.5 ml) containing 500 J2 was added to a microtube (1.7 ml volume). Then, 0.5 ml of each of the compounds skatole, γ -decalactone, 4-methylphenol or phenol, dissolved in 1% Tween-80 aqueous solution, was added to the microtube to achieve an LC_{50} of 106.02, 7.96, 70.81 and 18.68 mg l^{-1}, respectively, as reported in the literature (Pedroso *et al.*, 2019). Sterilised water and 1% Tween-80 were used as negative controls. Carbofuran at a concentration of 260 mg l^{-1}, corresponding to the LC_{50} reported in the literature (Oliveira *et al.*, 2014), was used as a positive control. The microtubes were kept at 28°C in an incubator for 48 h. Finally, the microtubes were opened, the contents removed and

added to 3 ml sterilised water. The total volume was then distributed in four holes (0.4 × 1.5 cm) made in the soil around a 30-day-old tomato 'Santa Clara' plant in a 300 ml plastic cup. The tomato plants were kept in a glasshouse for 40 days, receiving regular irrigation and fertiliser application. After this period, the plants were removed from the plastic cups. The roots were then washed free of soil, weighed, and the total number of galls was counted (infectivity). Then, the eggs were extracted from roots using a 0.5% NaOCl solution (Hussey & Barker, 1973), and an aliquot (1 ml) of each repetition was collected. The number of eggs was counted in a Peters chamber and extrapolated to the entire volume (reproduction).

MELOIDOGYNE INCOGNITA INFECTIVITY AND REPRODUCTION IN TOMATO PLANTS AFTER SOIL INOCULATION WITH J2 AND THE COMPOUNDS COMBINED

Tomato seeds 'Santa Clara' were planted in 300 ml plastic cups. Thirty days later, 1 ml of an aqueous suspension containing 500 J2 of *M. incognita* was added to 3 ml solutions of each compound, i.e., skatole, γ -decalactone, 4-methylphenol and phenol, to obtain the final concentrations of 100, 300 and 500 mg l^{-1}. Immediately afterward, the suspension was inoculated in four hole (0.4 × 1.5 cm) around each plant. Sterilised water and 1% Tween-80 were used as negative controls and carbofuran at 400 mg l^{-1} as a positive control. The tomato plants were kept in the glasshouse for 40 days, receiving regular irrigation and fertiliser application. After this period, the plants were removed from the plastic cups, their roots were washed and weighed, and *M. incognita* infectivity and reproduction were evaluated as described above.

IMMOBILITY AND MORTALITY OF *M. INCOGNITA* J2 EXPOSED TO VOLATILES OF THE COMPOUNDS

For this purpose, the method with glass flasks originally described by Barros *et al.* (2014a) and later modified by Jardim *et al.* (2018) was used. Therefore, 30 g of autoclaved dry sand (120°C for 20 min) were placed in Supelco® glass flasks (80 × 28 mm). Two microtubes of 0.6 ml each were inserted half-way into the sand of each flask. One of the two microtubes was filled with 100 µl of γ -decalactone, 4-methylphenol or phenol (liquids) or 100 mg of skatole (solid). The flasks were immediately sealed with screw caps, internally coated with a silicone film, and kept at 28°C in an incubator for 72 h. After this period, 0.5 ml of an aqueous suspension containing about 60 *M. incognita* J2 was injected into the empty microtube with a syringe. After 48 h at 28°C, the flasks were opened and the J2 transferred to

a 96-well polystyrene plate. The total numbers of mobile and immobile nematodes were quantified using an inverted objective microscope. Then, 1.0 M NaOH solution (20 µl) (Chen & Dickson, 2000) was placed in each well of the polystyrene plate to verify the movement of J2. Those that remained immobile were considered dead. The fumigant nematicide dazomet (100 mg) and sterilised water (100 µl) were used as positive and negative controls, respectively.

Effect of fumigation with compounds on *M. incognita* infectivity and reproduction in tomato plants

A 5 ml aqueous suspension containing 10 500 eggs of *M. incognita* was added to 1 l commercial growth substrate (Tropstrato®, Vida Verde Indústria e Comércio de Insumos Orgânicos) kept in a 2 l plastic bottle (Jardim *et al.*, 2018). The compounds skatole, γ-decalactone, 4-methylphenol and phenol were then distributed on the substrate at 200, 500 and 1000 µl (liquid) or mg (solid) (l substrate)$^{-1}$. The fumigant nematicide dazomet (250 mg l^{-1} substrate) was used as a positive control, while 1000 µl of sterilised water was used as a negative control. All bottles were sealed with PVC plastic film, and the resulting mixtures were homogenised by shaking the bottles and kept at 28°C for 3 days. The bottles were then opened and remained so for 5 days. After this period, the substrate from each bottle was transferred to five plastic cups of 200 ml volume. Thirty-day-old tomato plants 'Santa Clara' were transferred to the plastic cups containing the substrate and were kept in a glasshouse for 40 days. After this period, the roots were carefully washed and weighed. Then, *M. incognita* infectivity and reproduction were evaluated as previously described.

Statistical analysis

All experiments were repeated twice using a completely randomised design with five replicates. Except for the assays involving the infectivity and reproduction tests of inoculated J2 into the soil together with the compounds and of fumigation, no differences were observed among repeats of the same experiment. Therefore, a combined analysis was performed for each assay, and the data presented are the combined results of the repeat experiments. All results were submitted to normality (Shapiro- Wilk) and error variance homogeneity (Bartlett) tests. All data not fulfilling the normality and homogeneity pre-requirements were transformed. Thus, data on reproduction (first repetition) related to soil inoculation with J2

together with the compounds and related to the fumigation assay (both repetitions), as well as data on infectivity related to the fumigation assay (first repetition), were transformed to √x. In this way, the F-test was applied by ANOVA. When the F-test was significant ($P < 0.05$), the mean values for each treatment were grouped and differentiated by the Scott-Knott test. Sisvar (version 5.6) program was used for statistical analysis.

Results

In vitro hatching of *M. incognita* J2 from eggs exposed to the compounds

Hatching of *M. incognita* J2 was significantly different ($P < 0.0001$) between the compounds at the different concentrations. Phenol at all concentrations tested, and the lowest concentrations (100 mg l^{-1}) of γ –decalactone and 4-methylphenol, failed to affect hatching. However, all other compounds at all concentrations reduced J2 hatching compared with the negative controls (sterilized water and Tween-80). A concentration-related effect was observed with the compounds γ -decalactone, skatole and 4-methylphenol. γ-Decalactone and skatole at the concentrations of 300 and 500 mg l^{-1} and 4-methylphenol at 500 mg l^{-1} caused the highest reduction in the numbers of hatched J2 compared with Tween-80, i.e., the numbers were reduced by 42.1 to 59.3%. Skatole at 100 mg l^{-1} and 4-methylphenol at 300 mg l^{-1} reduced the number of hatched J2 by 28.7 and 27.5%, respectively (Table 1).

Meloidogyne incognita infectivity and reproduction in tomato plants after J2 exposure to the compounds at the LC_{50}

There was a significant effect of the compounds when *M. incognita* J2 were exposed to the respective LC_{50} values, both in terms of infectivity ($P < 0.0001$) and reproduction ($P < 0.004$). All compounds caused a reduction in the number of galls by 48.5 to 69.3% and in the number of eggs by 42.8 to 62.5% when compared with Tween-80, similar to carbofuran (data not shown).

Table 1. The number of *Meloidogyne incognita* second-stage juveniles (J2) hatching after contact of the eggs (500) with four compounds at different concentrations for 7 days.

Treatments	Concentration (mg l^{-1})	Hatched J2*
Water	-	69.8 c
Tween-80	10 000	69.8 c
Carbofuran	400	70.0 c
γ-Decalactone	100	66.3 c
γ-Decalactone	300	40.4 a
γ-Decalactone	500	30.6 a
Skatole	100	49.8 b
Skatole	300	39.9 a
Skatole	500	28.4 a
Phenol	100	71.2 c
Phenol	300	71.6 c
Phenol	500	71.2 c
4-Methylphenol	100	54.8 c
4-Methylphenol	300	50.6 b
4-Methylphenol	500	36.6 a

*Mean values followed by the same letter are not significantly different by the Scott-Knott test ($P < 0.05$). Data are the mean of ten replicates by joint analysis of two repeated experiments.

MELOIDOGYNE INCOGNITA INFECTIVITY AND REPRODUCTION IN TOMATO PLANTS AFTER SOIL INOCULATION WITH J2 AND THE COMPOUNDS COMBINED

There was a significant effect of the compounds at different concentrations on infectivity ($P < 0.0001$) and reproduction ($P < 0.0001$) of *M. incognita* (both repetitions) when compared with the negative controls (sterilised water and Tween-80). In both experiments, the greatest reductions in infectivity occurred with the highest concentrations of the compounds (500 mg l^{-1}) when compared to the negative controls, reducing the number of galls by 40.6 to 88.5% in the first experiment and by 21.8 to 34.2% in the second experiment. With regard to reproduction, all volatile compounds at all concentrations significantly reduced the number of eggs compared with the negative controls in both experiments. The highest concentration (500 mg l^{-1}) of each compound significantly reduced the egg number by 76.1 to 98.9% compared with the negative control (Tween® 80) in the first experiment and by 49.6 to 63.1% in the second experiment (Table 2).

Table 2. *Meloidogyne incognita* infectivity (galls (g root)$^{-1}$) and reproduction (eggs (g root)$^{-1}$) after the application of four compounds at different concentrations at the time of inoculation of second-stage juveniles in soil with tomato plants.

Treatments	Concentration (mg l^{-1})	Galls (g root)$^{-1}$*		Eggs (g root)$^{-1}$*	
		Experiment 1	Experiment 2	Experiment 1	Experiment 2
Water	-	20.4 c	38.9 c	2801.8 d	4962.6 d
Tween-80	10 000	21.7 c	38.0 c	2708.7 d	4877.0 d
Carbofuran	400	2.1 a	5.2 a	85.0 a	159.4 a
γ-Decalactone	100	22.8 c	37.4 c	1482.7 c	3082.9 c
γ-Decalactone	300	16.0 b	33.7 c	1561.7 c	3148.2 c
γ-Decalactone	500	4.3 a	29.7 b	573.8 b	2459.4 b
Skatole	100	22.9 c	37.1 c	2012.4 c	3041.6 c
Skatole	300	19.2 b	37.5 c	1164.4 c	3110.5 c
Skatole	500	12.9 b	25.0 b	646.8 b	2330.3 b
Phenol	100	17.8 b	36.9 c	412.5 b	3615.9 c
Phenol	300	17.1 b	36.6 c	415.7 b	3493.4 c
Phenol	500	2.5 a	28.4 b	29.0 a	1941.0 b
4-Methylphenol	100	24.4 c	38.0 c	1896.1 c	3457.4 c
4-Methylphenol	300	18.5 b	37.7 c	869.2 b	3397.4 c
4-Methylphenol	500	3.0 a	29.0 b	313.6 a	1800.0 b

*Mean values followed by the same letter are not significantly different by the Scott-Knott test ($P < 0.05$). Data are the mean of five replicates.

IMMOBILITY AND MORTALITY OF *M. INCOGNITA* J2 EXPOSED TO VOLATILES OF THE COMPOUNDS

There was a significant difference between the compounds in terms of *M. incognita* immobility (P <0.0001) and mortality (P < 0.0001) after exposure of J2 to their volatiles. Percentages of J2 immobility and mortality for phenol (95.8% for both immobility and mortality) and 4-methylphenol (100% for both immobility and mortality) were always higher than the positive control dazomet (87.6 and 80.2%). However, γ -decalactone failed to differ from the negative control (sterilised water) in any of the evaluations (immobility or mortality). Skatole was only significantly different from sterilised water when immobility was evaluated, and it caused only a small immobility percentage (4.7%) (Fig. 1).

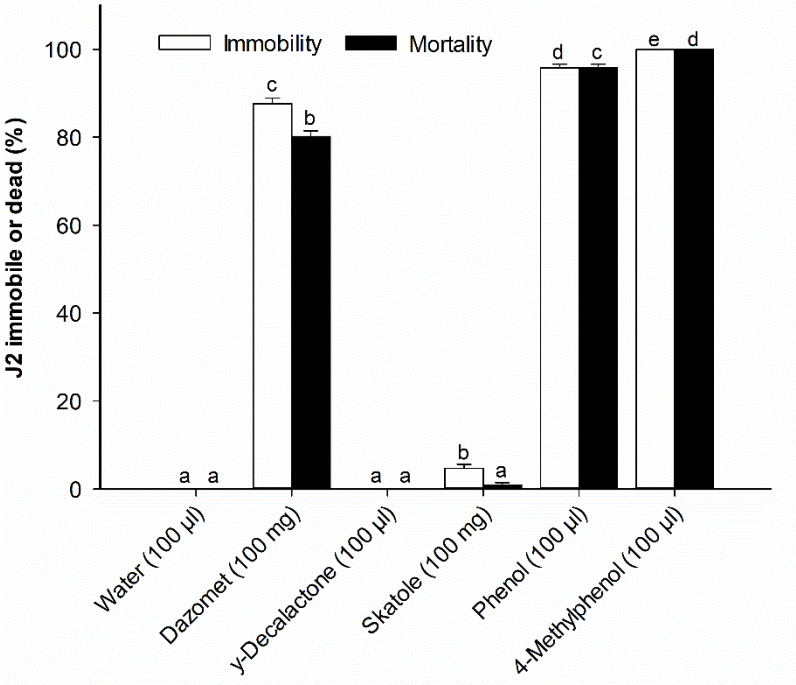

Fig. 1. Immobility and mortality of *Meloidogyne incognita* second-stage juveniles (J2) after exposure to volatiles of different compounds for 48 h. Sterilised water and Dazomet are controls. Mean values followed by the same letter are not significantly different by the Scott-Knott test ($P < 0.05$). Bars represent the standard error of the mean. Data are the mean of ten replicates by combined analysis of two repeated experiments.

EFFECT OF FUMIGATION WITH COMPOUNDS ON *M. INCOGNITA* INFECTIVITY AND REPRODUCTION IN TOMATO PLANTS

There was a significant effect of fumigation among the compounds at different concentrations when evaluating infectivity ($P < 0.0001$) and reproduction ($P < 0.0001$) of *M. incognita* (both experiments). With the exception of 4-methylphenol at 200 µl l^{-1} and γ -decalactone at 200 and 500 µl l^{-1} of substrate, all other concentrations of the compounds reduced the number of galls compared with the negative control (sterilised water) in both experiments. The highest concentrations (1000 mg or µl l^{-1} of substrate) of all compounds were responsible for the largest reduction in gall numbers when compared with the other concentrations (82.6 to 99.7%). This was similar to dazomet nematicide in the first experiment (98.7%) and close to its value in the second experiment (100%). All compounds at 1000 mg or µl l^{-1} showed greater reductions (87.1 to 99.7%) in egg numbers compared with the negative control in both experiments, but the highest reductions in egg numbers were observed with dazomet nematicide (98.6 and 100% in the first and second experiments, respectively) (Table 3). It is noteworthy that γ -decalactone at 200 and 500 µl l^{-1} was similar to sterilised water or caused significant increases in the number of galls and eggs, while 4-

methylphenol at 200 µl l^{-1} was always similar to sterilised water. None of the four compounds tested affected root development; only dazomet nematicide showed mass reduction (Table 4).

Table 3. *Meloidogyne incognita* infectivity (galls (g root)$^{-1}$) and reproduction (eggs (g root)$^{-1}$) after fumigation of the substrate containing eggs with four compounds at different concentrations.

Treatments	Concentration (l substrate)$^{-1}$	Galls (g root)$^{-1}$*		Eggs (g root)$^{-1}$*	
		Experiment 1	Experiment 2	Experiment 1	Experiment 2
Water	1000 µL	44.8 f	62.3 d	4806.2 d	5348.7 e
Dazomet	250 mg	0.6 a	0.0 a	65.6 a	0.0 a
γ-Decalactone	1000 µL	7.8 c	8.5 a	618.4 b	667.7 c
γ-Decalactone	500 µL	50.1 g	56.2 d	8287.6 e	5091.1 e
γ-Decalactone	200 µL	57.2 g	56.2 d	10761.9 f	5091.1 e
Skatole	1000 mg	1.6 a	5.2 a	350.6 b	264.5 b
Skatole	500 mg	32.2 e	47.3 c	2877.1 c	1583.1 d
Skatole	200 mg	29.1 e	49.9 c	3772.3 c	4510.0 e
Phenol	1000 µL	1.1 a	2.2 a	319.0 b	111.4 a
Phenol	500 µL	19.8 d	29.4 b	2792.3 c	1989.0 d
Phenol	200 µL	20.6 d	48.6 c	3189.0 c	5649.2 e
4-Methylphenol	1000 µL	4.8 b	0.2 a	392.1 b	18.7 a
4-Methylphenol	500 µL	22.8 d	22.8 b	2169.2 c	2468.5 d
4-Methylphenol	200 µL	41.1 f	58.8 d	5648.4 d	5764.4 e

*Mean values followed by the same letter are not significantly different by the Scott-Knott test ($P < 0.05$). Data are the mean of five replicates.

Table 4. The root mass of tomato plants after fumigation of the substrate containing eggs with four compounds at different concentrations.

Treatments	Concentration (l substrate)$^{-1}$	Root mass (g)*
Water	1000 µL	5.3 b
Dazomet	250 mg	2.0 a
γ-Decalactone	1000 µL	4.9 b
γ-Decalactone	500 µL	4.8 b
γ-Decalactone	200 µL	5.3 b
Skatole	1000 mg	5.6 b
Skatole	500 mg	5.2 b
Skatole	200 mg	5.2 b
Phenol	1000 µL	4.8 b
Phenol	500 µL	5.1 b
Phenol	200 µL	5.7 b
4-Methylphenol	1000 µL	5.0 b
4-Methylphenol	500 µL	5.3 b
4-Methylphenol	200 µL	5.3 b

*Mean values followed by the same letter are not significantly different by the Scott-Knott test ($P < 0.05$). Data are the mean of ten replicates by joint analysis of two repeated experiments.

Discussion

Processed oilseeds may generate promising residues against plant-parasitic nematodes, whether used in the field or in the VOCs production and identification. In addition, these compounds may result in the development of new nematicides (Chitwood, 2002).

Emphasis has been given to evaluating the toxic potential of the volatile compounds coming from complex emissions of plant tissue and their modes of action in plant-parasitic nematodes. In this study, the inhibition of hatching and the nematicidal and fumigant effects of four volatile compounds (γ -decalactone, skatole, phenol and 4-methylphenol) emitted by castor bean cake were evaluated. Such compounds have previously been shown to exhibit nematicidal effects *in vitro* against *M. incognita* J2 by direct contact (Pedroso *et al.*, 2019). The compound γ -decalactone has already demonstrated toxicity against other plant pathogens such as fungi and bacteria (Chambers *et al.*, 2013). Skatole inhibits biofilm formation, which may be regarded as a bacterial survival strategy, by enterohaemorrhagic *Escherichia coli* (Choi *et al.*, 2014), and in nematodes, weak activity has been reported against *Bursaphelenchus xylophilus* (Nagase *et al.*, 1982). Phenol and 4-methylphenol have not only been shown to be toxic to plant-pathogenic fungi and bacteria (Hwang *et al.*, 2005), but also they have *in vitro* nematicidal activity against plant-parasitic nematodes (Gu *et al.*, 2007; Huang *et al.*, 2010; Yang *et al.*, 2015); 4-methylphenol has also been shown to change the chemotaxis of the entomopathogenic nematode *Steinernema carpocapsae* depending on the cultivation temperature of the infective juveniles (Lee *et al.*, 2016). However, the *in vivo* activity of these compounds had not been evaluated previously.

The activities of volatile compounds based on direct toxicity to *M. incognita* J2 studied here have also been studied with several other compounds (Cheng *et al.*, 2017; Terra *et al.*, 2017; Zhai *et al.*, 2018). Although J2 is the infectious stage of root-knot nematodes, the effect of VOCs on eggs, which are the survival stage in the soil, may be considered. In the present study, the inhibition of hatching in *M. incognita* by VOCs from castor bean cake was evaluated and the compounds γ -decalactone, skatole and 4-methylphenol were effective. The lack of hatching inhibition by phenol does not disqualify it as a nematicidal compound, since evidence of nematicidal effects in J2 have already been reported in other studies (Gu *et al.*, 2007; Huang *et al.*, 2010; Pedroso *et al.*, 2019). In addition, eggs are more resistant to nematicides than J2 (Eisenback & Hunt, 2009). It should be emphasized that the nematicide carbofuran, well known on the market before its restriction, also failed to show inhibition of hatching in this study, corroborating the results obtained previously (Terra *et al.*, 2018).

A significant reduction in *M. incognita* infectivity and reproduction was also observed when J2 were in contact with the compounds at the LC_{50} values following inoculation. Some nematicidal compounds are active at concentrations well below their LC_{50}. In this case they do not cause death, but they may cause physiological or behavioural responses in plant-parasitic nematodes (Perry, 1996) preventing host recognition. When J2 were inoculated together with the compounds in different concentrations, there were significant reductions in infectivity and reproduction. Reductions in infectivity and reproduction of *M. incognita* were also verified when a combination of J2 and (*E*)-cinnamaldehyde were inoculated in soybean plants. The infectivity and reproduction were reduced by up to 100% and 99%, respectively (Jardim *et al.*, 2018). A 22% reduction in infectivity of *M. incognita* J2 was also observed when 2-methyl butyl acetate, emitted by a *Fusarium oxysporum* isolate, was applied to soil infested with *M. incognita* (Terra *et al.*, 2018).

In this study, we also verified the increase in immobility and mortality when J2 were exposed to volatiles of the compounds, with phenol and 4-methylphenol causing a high percentage in both evaluations. The action of (*E*)-cinnamaldehyde volatiles against *M. incognita* J2 was also investigated and found to cause 100% immobility and 84% mortality (Jardim *et al.*, 2018). The γ –decalactone and skatole compounds have higher boiling points than the other compounds here tested, and the lower the boiling point, the higher the volatilisation rate (Haydock *et al.*, 2013). This may have contributed to the lower percentages in the immobility and mortality tests.

Fumigant nematicides differ from the contact type because they add the action of vapour. Thus, in the fumigation process, the volatile action is of great importance as it affects the plant-parasitic nematode far away from the nematicide source (Chitwood, 2002). Moreover, fumigation is performed indoors, and there is no release of compounds into the atmosphere, which increases their efficiency (Haydock *et al.*, 2013). The volatiles of γ-decalactone and skatole were not effective in the *in vitro* tests when immobility and mortality of *M. incognita* J2 were evaluated. However, they caused a high reduction in the numbers of galls and eggs when used as fumigants at the highest concentrations tested against eggs in the soil, as did the other compounds tested, with an effect similar to that of dazomet nematicide. Moreover, the compounds may be used in substrate disinfestation for transplanting and seeding. Although some plant-derived compounds may exhibit phytotoxic effects depending on the concentration used (Roh *et al.*, 2011), none of the four compounds tested against plant-parasitic nematodes here affected plant development, which supports the conclusion that these are not phytotoxic substances.

Although γ -decalactone was effective in fumigation of the substrate at the highest concentration, the lower concentrations were similar to sterilised water or favored gall formation and consequent egg production. Other compounds also elicit different nematode responses depending on the concentration used. In a study of *M. incognita* chemotaxis towards VOCs, acetone, 2-decanol and furfural acetone attracted *M. incognita*, and 2-undecanone acted as a repellent. However, 4-acetylbenzoic acid was shown to be an attractant at a low concentration but repellent at a high concentration (Cheng *et al.*, 2017).

With regard to the different concentrations of compounds used in this study, either to investigate the inhibition of hatching and the effect on infectivity and reproduction (100, 300 and 500 mg l^{-1}), or to investigate the fumigant effect (200, 500 and 1000 mg or µl l^{-1} of substrate), the highest concentrations were always the most effective. The hatching reduction and nematicidal effects of compounds at 500 mg l^{-1} in this study are corroborated by other results that reported inhibition of hatching of *M. incognita* by 3-methyl butyl acetate and ethyl acetate compounds. In addition to these compounds, 2-methyl butyl and 2-methyl propyl acetate at this concentration also caused a reduction in the infectivity and reproduction of *M. incognita* when in contact with eggs followed by inoculation in soil with tomato plants (Terra *et al.*, 2018). The best fumigant efficacy at the highest concentration (1000 µl (l substrate)$^{-1}$) of all compounds tested in this study is also corroborated by another study that reported the efficacy of (*E*)-cinnamaldehyde at the same concentration in the reduction of galls and eggs – greater than with other concentrations (200 and 500 µl l^{-1}) (Jardim *et al.*, 2018).

In general, 4-methylphenol stood out by presenting different modes of action: inhibition of hatching, nematicidal activity involving a reduction in infectivity and reproduction, and fumigant action in the *in vitro* test against J2 and in the *in vivo* test against the eggs. In another work, 4-methylphenol showed the highest efficiency, with 100% mortality occurring at only 30 mg l−1 (Yang *et al.*, 2015). On the other hand, in this study, phenol did not cause inhibition of hatching, but it showed nematicidal activity with fumigation, as did γ -decalactone and skatole. These last compounds, in turn, inhibited hatching but did not have fumigant activity *in vitro* against J2. Not all compounds are effective against nematodes by different modes of action. Among seven VOCs emitted by an isolate of the bacterium *Pseudomonas putida*, five had strong nematicidal activity against *M. incognita* J2 by direct contact, but only one acted as a fumigant. In addition, the seven VOCs inhibited *M. incognita* hatching by both direct contact and fumigation (Zhai *et al.*, 2018). Eleven VOCs emitted by an isolate of the bacterium *Paenibacillus polymyxa* were investigated in the control of *M. incognita*. Of these, eight had nematicidal contact activity,

six had fumigant activity and five acted as chemotactic agents to *M. incognita*, of which two attracted *M. incognita* J2 and then killed it by direct contact or fumigation (Cheng *et al.*, 2017). However, none of these studies included the compounds studied here.

In conclusion, there is no doubt about the potential of the compounds emitted by castor bean cake – γ -decalactone, skatole, phenol and 4-methylphenol – for the development of new products to control root-knot nematodes, since these compounds act as nematicides in several ways in the *M. incognita* life cycle.

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ARTIGO 2 – Nematicidal activity of ethanol solutions on soybean cyst nematode *Heterodera glycines*

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Elaborado de acordo com as normas do periódico Nematology.

Summary – The cyst nematode, *Heterodera glycines*, is a major pathogen of soybean in tropical regions, which demands novel sustainable practices. In this work, the use of ethanol against *H. glycines* was evaluated as both a solution and a fumigant. On second-stage juveniles (J2) of *H. glycines*, ethanol at low concentration was more effective by direct dipping than by only fumigating the J2. Hatching was significantly reduced by direct dipping in ethanol solutions. Fumigation of *H. glycines*-infested soil with ethanol reduced infectivity by almost 100% and the number of eggs by about 67% at ethanol concentrations of 48% and 72%, respectively. Only the ethanol at 48% concentration significantly reduced the J2 lipid content, while J2 infectivity and the number of eggs were reduced by dipping at 6% ethanol. The J2 were internally altered by the ethanol solutions. Therefore, ethanol is toxic to *H. glycines* at low concentrations and affects its pathogenic behaviour rather than simply reducing the lipids.

Keywords – alcohol, alternative control, *Glycine max*, organic farming, plant-parasitic nematodes, soil pathogens, soybean.

Soybean (*Glycine max* (L.) Merr.) is a crop of great economic importance worldwide and is one of the most important vegetable sources of protein and oil in several countries (Schmutz, *et al*., 2010). The USA is the largest producer of soybean, followed by Brazil (USDA, 2018), which produced more than 119 million tons of grains in the 2017/2018 season (CONAB, 2018). In addition to conventional soybean cropping, there has been an expansion in organic soybean cropping recently due to demand for healthy and sustainable farming. The high values of organic soybean have attracted new farmers and consumers in the last few years, as similar yields and lower costs are possible with organic soybeans compared to conventional soybeans (McBride & Greene, 2009). However, there are still limiting factors of yield, both in conventional or organic systems, such as biotic diseases (Lima *et al*., 2008). The search for novel practices to control soybean diseases and maintain environmental preservation has been continuous. However, new methods may be costly for farmers (Hartman *et al*., 2016). Among the diseases of soybeans, which include those caused by plant-parasitic nematodes, one of the most severe is the soybean cyst nematode, *Heterodera glycines*, which has caused an estimated annual loss in soybean yield of approximately 1.5 billion US$ in the USA alone (Han *et al*., 2015). Strategies to reduce important nematodes, such as *H. glycines*, and maintain a sustainable and profitable production level are still far from reality for small, organic soybean farmers (Ito *et al*., 2015; Lee & Choe, 2019).

One alternative to manage plant-parasitic nematodes in an ecologically friendly way is the application of natural compounds from plants or microorganisms (Terra *et al*., 2018; Pedroso *et al*., 2019; Silva *et al*., 2019). Several alcohol compounds are produced as part of the secondary metabolisms of live organisms (Larsen & Frisvad, 1995). Ethanol is non-toxic to animals and humans, it quickly evaporates after application and it has been used as a disinfectant or biocide in 70% aqueous solution by acting as an organic solvent (Mishra, 1993). Plant pathogens could be controlled by the use of ethanol solutions, which have been shown to act efficiently against fungi, bacteria and nematodes in different systems (Lichter *et al*., 2002; Romanazzi *et al*., 2012; Silva *et al*., 2017). Most of the studies show the effects against plant pathogens by fumigation (Silva *et al*., 2017; Yuen *et al*., 1995; Utama *et al*., 2002). However, direct application of ethanol has also been shown to be highly toxic to plant-parasitic nematodes (Silva *et al*., 2017).

The direct contact of nematodes with ethanol solutions decreases their reproduction and movement and consequently hinders their normal activities and growth (Dhawan *et al*., 1999; Davis *et al*., 2008). There is strong evidence of ethanol interfering with the nervous systems of nematodes at low concentrations (Morgan & Sedensky, 1995; Davies *et al*., 2004).

Most of the ethanol studies have been performed with the free-living nematode *Caenorhabditis elegans* (Dhawan *et al*., 1999; Davis *et al*., 2008). Nevertheless, nematicidal effects against *Meloidogyne incognita* have been reported when applying ethanol solutions at low concentrations directly or by fumigation (Silva *et al*., 2017). However, little is known concerning ethanol effects against other important plant-parasitic nematodes, and there are no studies with nematodes on the possible effects of ethanol as an organic solvent, rather than acting by some other mechanism.

It is well known that lipids are the main energy source of plant-parasitic nematodes until they reach and infect their host (Lee & Atkison, 1977; Van Gundy, 1985). Stored energy, as measured by lipid content, is extremely important to nematode parasitism and life cycle completion. Therefore, to understand the possible effects of ethanol on lipid content, the ethanol toxicity against plant-parasitic nematodes should be clarified and the practicability in field conditions improved.

Despite some studies of the action of ethanol against root knot nematodes (Silva *et al*., 2017), there are no reports of ethanol applied against *H. glycines*, and ethanol could be promising for use in soybean organic production. Therefore, this study evaluated the toxicity of aqueous solutions of ethanol by direct dipping or by fumigation against *H. glycines* under laboratory and soil conditions. First, we determined the effective toxic concentrations of ethanol against second-stage juveniles (J2) and eggs of *H. glycines*. Then, by fumigation of *H. glycines* infested soil with ethanol, we estimated the effect of different concentrations on the infection and number of eggs. Finally, we verified the effect of ethanol at different concentrations on J2 lipid content and the consequences on the infectivity of the J2.

Materials and methods

OBTAINING CYSTS, EGGS AND J2 OF *H. GLYCINES*

Heterodera glycines cysts were obtained from pure population multiplied on soybean plants (*G. max* ‘M6410IPRO’) kept under glasshouse conditions. Cysts were extracted from soil samples (100 cm^3) following the decantation method (Chen *et al*., 2001) using 20- and 100-mesh sieves. To obtain eggs, the cysts were mechanically crushed on a 100-mesh sieve placed over a 200-mesh and a 500-mesh sieve. The material retained in the last sieve was collected and placed in a hatching chamber at 25°C in the dark to obtain the J2. We only used J2 that hatched after 24 h.

DIRECT DIPPING AND FUMIGATION OF *H. GLYCINES* J2 WITH ETHANOL SOLUTIONS

To evaluate the nematodes in direct contact with ethanol, J2 of *H. glycines* were dipped in aqueous solutions of ethanol. Each ethanol solution (0.5 ml) was placed into microtubes (1.7 ml capacity), together with a 0.5 ml aqueous suspension containing 60 J2 (Fig. 1A). The final solution corresponded to half the original concentration of ethanol, resulting in concentrations of 1.5, 3.0, 6.0, 12.0, 24.0 and 48.0% (ethanol/water, v/v). As a control, sterilised water was placed in a microtube with the same volume of each solution (0.5 mL sterilised water and 0.5 mL of J2 suspension). The microtubes were sealed with PVC plastic film and incubated at 25°C for 24 h. To evaluate the fumigation with ethanol, we adapted Supelco® glass vials (80 × 28mm; Sigma-Aldrich) (Barros *et al*., 2014a). Dry sand (30g) that had been autoclaved at 120°C for 20 min was placed inside the vials to support two microtubes (0.6 ml capacity) that were inserted next to each other halfway into the sand. The ethanol solution (0.5 ml) was placed in one microtube and an aqueous suspension (0.5 ml) containing 60 nematodes was placed in the other microtube (Fig. 1B). The vials were then sealed and incubated at 25°C for 24 h after which the microtubes and vials were opened and the J2 were washed on a 500-mesh sieve with distilled water. Then, the J2 were transferred to five wells (0.4 ml capacity) of a 96-well polystyrene plate. Using an inverted objective microscope, the mobile and immobile nematodes were quantified and the percentage of immobile J2 was obtained. To determine if the effect was reversible, the wells of the plate containing the nematodes were filled with distilled water and incubated again at 25°C for 24 h, and the percentage of killed J2 was finally determined by considering the J2 still immobile to be dead.

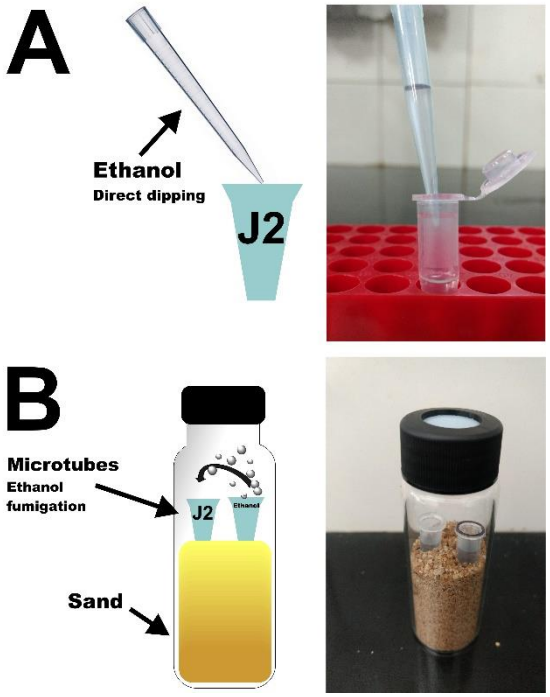

Fig. 1. Procedures to evaluate the effect of ethanol *Heterodera glycines* second-stage juveniles (J2). A: direct dipping in ethanol solutions using microtubes; B: fumigation with ethanol solutions using vials filled with sand to support two microtubes, one with ethanol solution and other with J2.

DIRECT DIPPING AND FUMIGATION OF *H. GLYCINES* EGGS WITH ETHANOL SOLUTIONS

For tests on nematode eggs, ethanol solutions were prepared at the same concentrations as in the previous assay. In addition, the same methodology was used, but an aqueous suspension (0.5 ml) containing 1000 eggs of *H. glycines* was substituted for the J2 suspension. The microtubes and vials were then sealed and incubated at 25°C for 7 days, after which the microtubes and vials were opened, the suspension was washed on a 500-mesh sieve with distilled water and the number of hatched J2 was quantified using a light microscope. Next, the suspension containing the remaining eggs and hatched J2 was transferred to fresh distilled water. The suspension was kept at 25°C for five more days in the water; the number of hatched J2 was quantified again.

SOIL FUMIGATION WITH ETHANOL AGAINST *H. GLYCINES*

Eggs of *H. glycines* (15,000) were added to 1l of previously autoclaved (120°C for 60 min) soil and sand (1:1) and placed in individual plastic PET bottles (2 l) (Jardim *et al*., 2018). Aqueous ethanol solutions (20 ml) were then added at concentrations of 0, 12, 24, 48, 72 and 96%; distilled water only was added (20 ml) to the 0% concentration (control). The

soil and ethanol were mixed by shaking the bottle. All bottles were sealed with PVC plastic film and remained at 25°C for 3 days. The bottles were then opened and remained so for another 3 days. After this period, the mixture from each bottle was deposited into five plastic cups (200 ml), constituting the replicates. Thirty-day soybean plants (*G. max* 'M6410IPRO') susceptible to *H. glycines* were transferred to the plastic cups with the mixture and kept in a glasshouse for 35 days, receiving adequate irrigation and fertiliser application. After this period, the numbers of cysts and females in each root system and in the soil (infectivity) were evaluated. Then, ten cysts of each plant were collected following the decantation method (Chen *et al*., 2001). To obtain eggs and J2, the cysts were mechanically crushed on a 100-mesh sieve placed over a 200-mesh and a 500-mesh sieve. The material retained in the last sieve was collected and placed in a Peters chamber to estimate the number of eggs and J2 per cyst with an inverted microscope.

THE INFLUENCE OF ETHANOL ON THE LIPID CONTENT OF *H. GLYCINES* J2 AND ITS EFFECT ON INFECTIVITY AND EGGS FORMATION

An aqueous suspension (0.5 ml) containing 60 J2 was placed in a microtube (1.7 ml capacity) together with the ethanol solutions (0.5 ml), giving resulting ethanol concentrations of 6, 12 and 48%. As a control, sterilised water was filled to the same volume. Microtubes were sealed with PVC plastic film and incubated at 25°C for 24 h. To compare the neutral lipids of the *H. glycines* dipped in ethanol, freshly hatched J2 were placed in microtubes containing water (control) to give maximum lipid content. Suspensions containing the J2 were then concentrated (0.5 ml), and the supernatant was discarded. Oil Red O dye solution (3 ml) was added (Storey, 1983; Christophers *et al*., 1997). Dye solution was prepared using Oil Red O (0.5 g) and absolute alcohol (100 ml), stirred for 15 min with a magnetic stirrer and then filtered using standard filter paper. The solution was stored in a refrigerator at 5°C in a flask stored in the dark. After addition of the Oil Red O dye to the J2, the suspension was heated in a water bath at 60°C for 20 min. After completely cooling to room temperature (about 25°C), the supernatant was discarded and the nematodes were again concentrated (0.5 ml). A solution (3 ml) of distilled water and pure glycerin (1:1) was then added and the suspension was stored. Five replicates were prepared for each treatment, and for each replicate, a slide containing six nematodes was prepared. Images of each nematode were obtained enabling measurements of the total area of the nematode body and the area coloured red, which corresponded to the lipids. For this, the software Assess® (American Phytopathological

Society, St. Paul, MN, USA) was used. The mean lipid content of six nematodes was obtained for each replicate.

To evaluate the infectivity and eggs formation of *H. glycines* J2 dipped in ethanol solutions for 24 h, 30-day-old soybean plants were planted in plastic cups (300 ml) containing autoclaved (120°C for 60 min) soil and sand (1:1). The assay was prepared using the same technique as for the neutral lipids content, dipping J2 into 6, 12 and 48% ethanol solutions. However, instead of 60 J2, 500 J2 were used. After 24 h, the J2 were washed on a 500-mesh sieve with distilled water, and 3 ml of water were added to the suspension containing the nematodes, totalling 4 ml. The suspension was distributed among four holes (1.5 cm depth) around a soybean seedling (*G. max* 'M6410IPRO'). The seedlings were kept in a glasshouse, and they received adequate irrigation and fertiliser applications. Thirty-five days after inoculation, the infectivity and number of eggs were evaluated as previously described for the soil fumigation experiment.

STATISTICAL ANALYSIS

All the assays had five replicates and were repeated twice, except for the evaluation of the percentage of lipids in the nematodes, since for each repetition, the mean of six nematodes was used. The assays were performed using a completely randomised design. All the experiments that were performed twice were submitted to a factorial scheme, and once they presented no differences ($P > 0.05$), we performed joint analyses by analysing all replicates together. All results were previously submitted to normality (Shapiro-Wilk) and error variance homogeneity tests (Bartlett). Data that failed to fulfil normality and homogeneity pre-requirements were transformed. Thus, data on infectivity related to fumigation in addition to data on eggs formation after exposure the J2 to ethanol were transformed by $\sqrt{x}$. Two-way ANOVAs were performed in all *in vitro* assays to evaluate the ethanol solutions or vapours, with a factorial scheme of contacts × concentrations. One-way ANOVAs were performed for the remaining experiments. The means of each treatment were grouped by the Tukey test at a 5% significance level, or regression analyses were performed and the most suitable models for the curves were adjusted.

Results

DIRECT DIPPING AND FUMIGATION OF *H. GLYCINES* J2 WITH ETHANOL SOLUTIONS

There was a significant interaction ($P < 0.001$) between the increase of the concentration and the ethanol application method (direct dipping or fumigation), when evaluating the immobility or mortality of the *H. glycines* J2. Immobility reached 100% when the nematodes were direct dipped in all the ethanol aqueous solutions from 6% concentrations upwards. By contrast, 72.9% immobility was reached in the ethanol fumigation only by using 24% ethanol, and reached 100% immobility after applying the 48% solution (Fig. 2A). By direct dipping in the ethanol solutions, 98.1% of J2 died from 12% ethanol, while by ethanol fumigation only 19.8% of J2 died by applying the highest concentration of 48% (Fig. 2B).

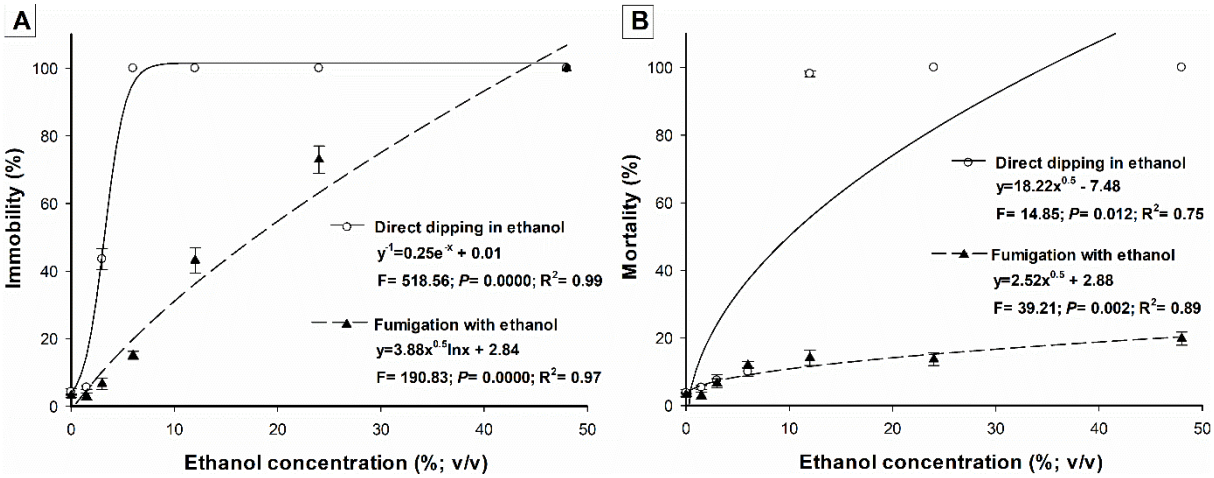

Fig. 2. Immobility (A) and mortality (B) of *Heterodera glycines* second-stage juveniles after direct dipping in aqueous solutions of ethanol or by fumigation at different concentrations of ethanol for 24 h. Bars represent standard error. Data are the mean of ten replicates by joint analysis of two repeated experiments.

DIRECT DIPPING AND FUMIGATION OF *H. GLYCINES* EGGS IN ETHANOL SOLUTIONS

There was a significant interaction ($P < 0.001$) between the increase of the concentration of ethanol and the ethanol application method (direct dipping or fumigation), when evaluating hatching of *H. glycines*, either after ethanol exposure or after subsequent 5 days in distilled water. In general, the reduction of hatched J2 by direct dipping in ethanol was greater than by fumigation. When direct dipped in ethanol, 1.5% ethanol reduced hatching by 72.2% in both evaluations. The fumigation with 6% ethanol reduced the number of hatched J2

by 66.3% in the first evaluation, whereas, in the second evaluation, the same ethanol solution reduced hatching by 56.5% (Fig 3).

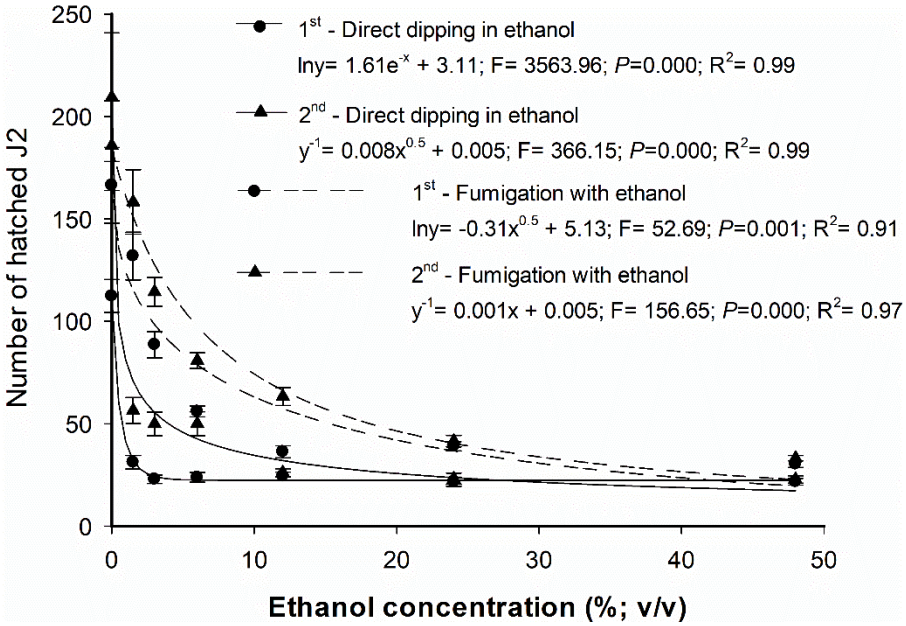

Fig. 3. The effect of ethanol on hatching of *Heterodera glycines* after direct dipping or fumigation at different concentrations for 7 days. The second-stage juveniles (J2) were quantified after removing the eggs from microtubes and washing them with distilled water (1st evaluation). After 5 days, the J2 were quantified again (2nd evaluation). Bars represent standard error. Data are the mean of ten replicates by joint analysis of two repeated experiments.

SOIL FUMIGATION WITH ETHANOL AGAINST *H. GLYCINES*

When fumigation of soil containing *H. glycines* eggs was performed by applying aqueous solutions of ethanol, there was a significant reduction in the number of cysts/females ($P < 0.001$) and eggs/J2 ($P < 0.001$). The number of cysts/females per cup was significantly reduced by an application of 12% ethanol, and with 48% ethanol, the reduction reached 96.0%. The number of eggs/J2 per cyst was reduced by 66.7% when ethanol at 72% concentration was applied (Fig. 4).

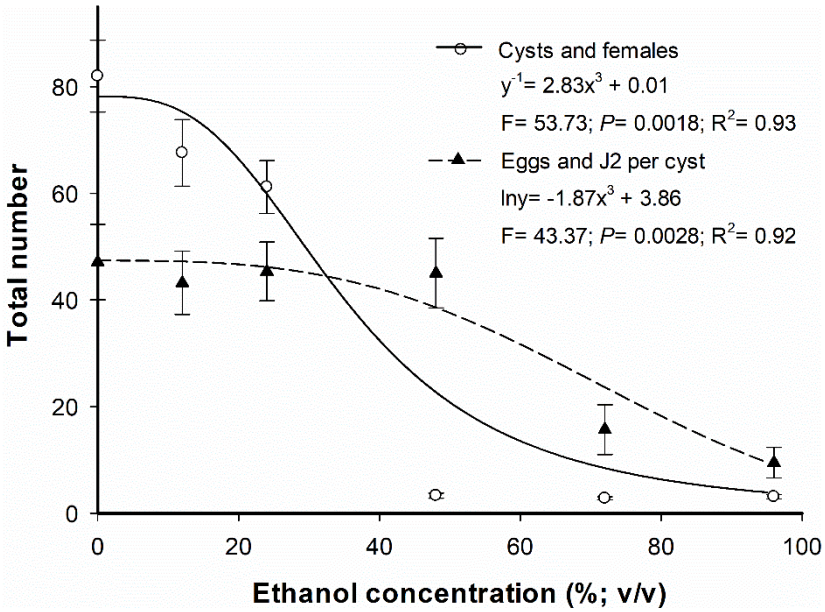

Fig. 4. Infectivity (cysts and females) and reproduction (eggs and second-stage juveniles (J2)) of *Heterodera glycines* after soil fumigation with ethanol. Eggs and ethanol at different concentration were mixed before planting soybean seedlings. Bars represent the standard error. Data are the mean of ten replicates by joint analysis of two repeated experiments.

ETHANOL INFLUENCE ON THE LIPID CONTENT OF *H. GLYCINES* J2 AND ITS EFFECT ON INFECTIVITY AND EGGS FORMATION

There was a significant difference ($P < 0.01$) in neutral lipids (percentage of total red coloured areas). By applying the 48% ethanol solution the percentage of lipids was reduced about 17.0% when compared to the freshly hatched J2, similarly to the control (0% ethanol) reduction, which maintained the mobility and naturally loss of lipid content. However, the ethanol concentrations of both 6% and 12% failed to reduce the lipid content (Fig. 5A). The distribution of lipid content in the J2 at 6% and 12% ethanol concentrations was slightly altered (Figs. 5D, E). However, when the J2 were dipped at 48% ethanol concentration, the disorganisation of lipid distribution throughout the nematode was noticeable and showed extensive vacuolisation (Fig. 5F).

There was a significant reduction ($P < 0.001$) in numbers of both cysts/females and eggs/J2 of *H. glycines*, when inoculating J2 that had been dipped in 6% and higher ethanol solutions. For example, the number of cysts/females was reduced by 96.0% and the number of eggs/juveniles was reduced by 95.2% by the 12% ethanol concentration (Fig. 6).

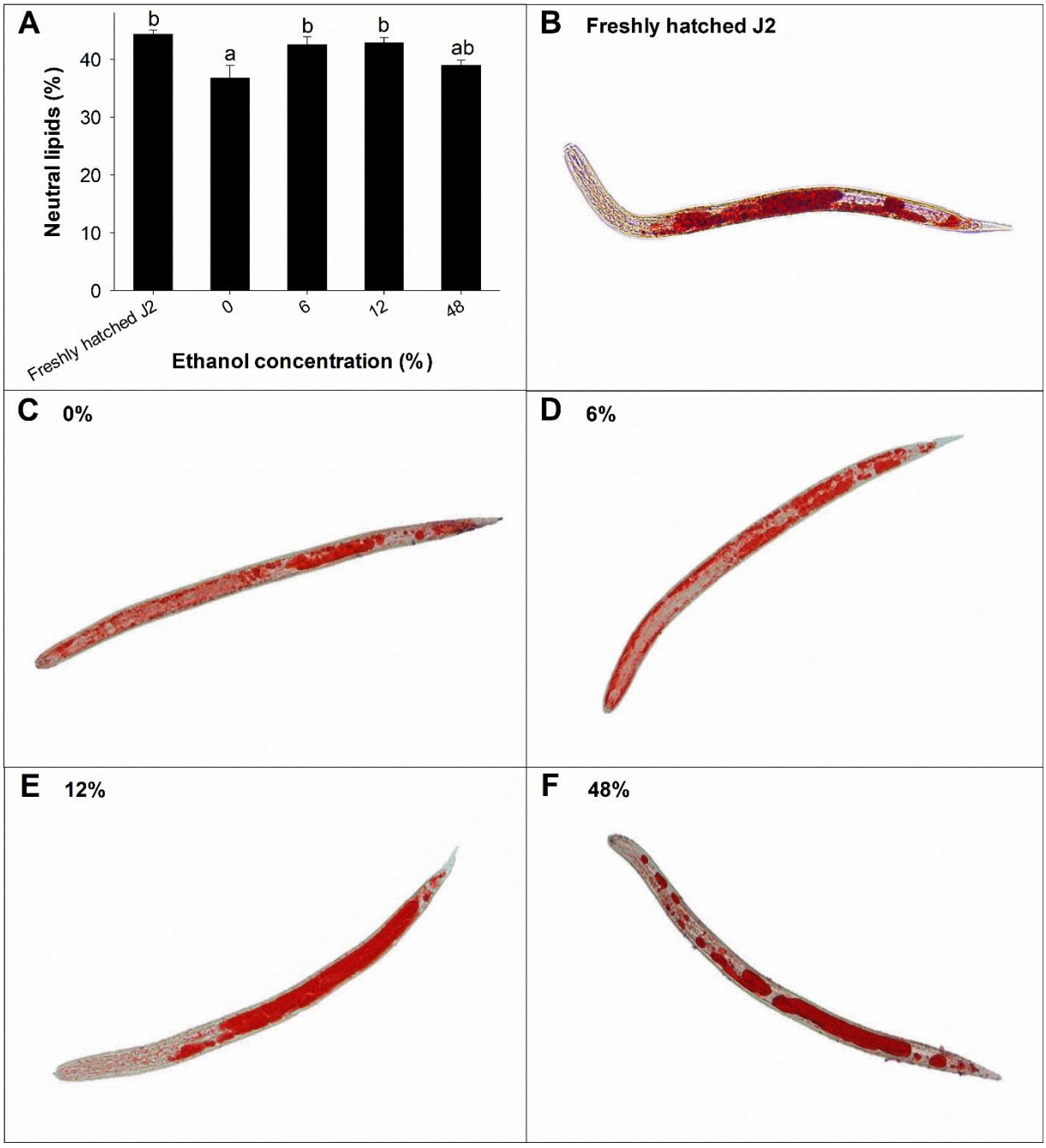

Fig. 5. Influence of ethanol on lipid contents of *Heterodera glycines* second-stage juveniles (J2). A: Percentage of neutral lipids in freshly hatched J2 compared to J2 directly dipped into aqueous solutions of ethanol at different concentrations for 24 h. Mean values followed by the same letter did not differ significantly from each other by Tukey's test ($P < 0.05$). Bars represent the standard error. Data are the mean of five replicates. B-F: Lipid content stained with Oil Red O of freshly hatched J2 or after direct dipping in ethanol solutions.

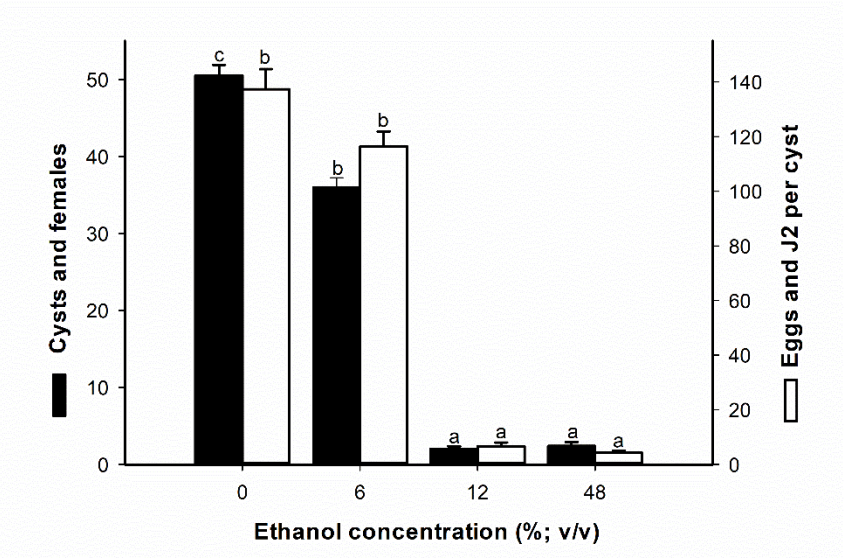

Fig. 6. Infectivity (cysts and females) and reproduction (eggs and second-stage juveniles (J2)) of *Heterodera glycines* after dipping the J2 into aqueous solutions of ethanol at different concentrations for 24 h. Mean values followed by the same letter did not differ significantly from each other by Tukey's test ($P < 0.05$). Bars represent the standard error. Data are the mean of ten replicates by joint analyses by joint analysis of two repeated experiments.

Discussion

This work provides evidence of the nematicidal effects of ethanol against *H. glycines* by direct dipping or fumigation. In general, ethanol solutions were more effective against *H. glycines* by direct dipping than by fumigation, even at low concentrations. The toxic effects were supported by evaluations of immobility, mortality, hatching, and infectivity and eggs formation in soybean plants. Soil fumigation with ethanol reduced cysts/females and eggs/J2, and high ethanol concentrations affected the neutral lipids of *H. glycines* J2. The application of ethanol to control plant pathogens has been previously reported as a feasible strategy either by direct contact or by fumigation (Lichter *et al*., 2002; Utama *et al*., 2002; Karabulut *et al*., 2003; Romanazzi *et al*., 2012; Silva *et al*., 2017). In addition, exposure to extreme conditions or to certain molecules is known to change the lipid content of plant-parasitic nematodes (Campos *et al*., 2006; Rocha *et al*., 2009) as well as entomopathogenic nematodes (Andaló *et al*., 2011).

The effects of ethanol have been studied mostly against the nematode *C. elegans* (Dhawan *et al*., 1999; Davis *et al*., 2008). The negative effects of ethanol on *C. elegans* started after exposure of the nematode to a 5% ethanol solution, which caused low mobility and interfered in nematode reproduction (Yu *et al*., 2011). In this work, the direct dipping of *H. glycines* with ethanol at low concentrations caused marked immobility and mortality,

whereas fumigation did not kill a large number of *H. glycines* J2. However, other research has reported that the effects of ethanol on the root-knot nematode *M. incognita* were the same by direct dipping or by fumigation (Silva *et al*., 2017). The cuticle of *H. glycines* J2 is thicker than the cuticle of *M. incognita* J2 (Eisenback, 1985; Zunke & Eisenback, 1998), consequently the ability of ethanol to penetrate the cuticle layers may be lower for *H. glycines* than for *M. incognita*, since the cuticle is responsible for controlling the permeability of many compounds (Davies & Curtis, 2011).

The aqueous ethanol solutions reduced *H. glycines* hatching significantly at very low concentration (from 1.5%) by direct dipping and at 6% and above by fumigation of ethanol; the effect was irreversible even after 5 days in distilled water. This suggests that the ethanol solution crosses through the chitin and lipid layers of the egg. Such layers are responsible for embryo and juvenile protection inside the egg (Perry & Trett, 1986). In addition, before embryo formation, multiple cell stages can also suffer the effects of ethanol. One of the activities of ethanol against microorganisms is against the cell membrane by affecting its structure and biological functions (Dao & Dantigny, 2011). Furthermore, there was no difference between the first and second evaluations of hatching (Fig. 3); this indicates the long-lasting effect of ethanol in either direct dipping or fumigation.

Ethanol is widely used as an organic solvent in plant extracts applied to the soil as nematicides (Dawar *et al*., 2007; Tariq *et al*., 2007). Although ethanolic extract usually damages more nematodes than aqueous extract, the exclusive effect of ethanol has not been extensively verified (Babaali *et al*., 2017). This study showed a significant reduction in the number of cysts and females, as well as in the number of eggs and J2 per *H. glycines* cyst after soil fumigation with ethanol solutions, mainly at 48% ethanol concentration. The number of galls and eggs of *M. incognita* was also reduced with the application of low doses of 40% ethanol concentration in infested soils (Silva *et al*., 2017). Again, our results reinforce the possibility that the cuticle thickness of *H. glycines* interferes in the permeability and, consequently, on the effects of ethanol when compared to the cuticle of *M. incognita*.

Once inside the J2, the ethanol may damage the internal components of *H. glycines*. The body of each J2 consists of about 25% lipids, which represent the main energy storage. The lipids are strongly linked with the parasitism activity of nematodes (von Mende *et al*., 1998). When moving though soil pores, the J2 utilise part of their lipids, decreasing their infective ability. In the present study, nematodes in the control (without ethanol) showed a reduced percentage of lipid content after 24 h, which did not occur after direct dipping with 6% and 12% concentrations of ethanol. By applying ethanol to J2, the nematode stopped

moving or died, thus preserving the lipid contents. When J2 of *Globodera rostochiensis* were exposed to the nematicide oxamyl for 8–35 days, the final lipid content was significantly higher than controls kept in water (Wright *et al*., 1989). The lipid content of J2 of *H. oryzae* was reduced by 70% after storage in water for 5 weeks (Reversat, 1980), and infectivity was closely associated with lipid losses (Reversat, 1981). The 6% ethanol did not kill, but it immobilised the J2 of *H. glycines*, which kept the lipid content at the same level as that of freshly hatched J2. Exposure to 6 and 12% ethanol resulted in the distribution of lipid throughout the J2 becoming disorganised showing areas without lipids. However, by applying ethanol solutions at 12 and 48%, the J2 died, but we noticed a reduction of the lipid content only with 48% ethanol, where the lipid became disorganised and appeared vacuolated (Fig. 5F). Organic compounds at certain concentration may not dissolve the lipid, but interfere in its use in the metabolism, thus damaging the nematodes. When J2 of *M. incognita* were maintained in erucin solution for 24 h at 10 mg l^{-1}, an abnormal vacuolisation was also observed inside the nematodes in addition to decreased J2 activity (Aissani *et al*., 2015). Therefore, similarly to erucin, ethanol appears to act as an organic solvent, disorganising the lipids inside the nematodes. In the present work, the infectivity and number of eggs of *H. glycines* were reduced after the J2 remained in contact with the ethanol at low concentrations, even when it did not kill them. Many studies have reported the correlation of immobility with infectivity and reproduction (Barros *et al*., 2014b).

Nematodes need water to move into the soil and the ethanol is easily diluted in water. Thus, the use of ethanol in irrigated areas should be effective, since this method is easy to apply for nematode control (Momma *et al*., 2013; Hewavitharana *et al*., 2014; Silva *et al*., 2017). In addition, the impact of this technique on native beneficial microorganisms is considered low, promoting a temporary increase in the number of anaerobic bacteria, while the number of fungi and aerobic bacteria remains stable (Momma *et al*., 2010). In this study, 6% ethanol was toxic to *H. glycines* and consequently reduced J2 infectivity and the resulting number of cysts and females. Ethanol is usable mostly in small areas, such in organic soybean fields. Lately, organic soybeans have been adopted as alternatives to replace other crops or even the conventional soybeans, due to high yields and low production costs (McBride & Greene, 2009). Therefore, based on our results, 1.8 m^3 of absolute ethanol (99.5%) ha^{-1} should be applied, to obtain a concentration of 6% in an aqueous solution. Because the nematodes tend to occur in clusters in the field, if precision management strategies are used in the infested areas, the cost-benefit ratio will certainly be favourable, since this technology allows the maximisation of control and sustainability of the productive system (Soares *et al*., 2016).

Another option is to use agro-industry residues that contain ethanol in their composition, such as sugarcane stillage, which has approximately 8.5% ethanol, and has shown to be effective in the control of plant-parasitic nematodes (Pedrosa *et al*., 2005; Leite *et al*., 2019). This residue, usually applied for other purposes, could improve the nematode management in soybean fields.

In summary, in this work we found that aqueous solutions of ethanol are more toxic to *H. glycines* by direct dipping than fumigation. Furthermore, the toxic effects began with application of 6% ethanol solutions to *H. glycines* J2 and eggs. Soil fumigation with a 6% concentration of ethanol also reduced the number of cysts/females and eggs/J2, which implies a reduction of infectivity and reproduction, creating the prospect of using ethanol as a feasible practice to control *H. glycines*. Finally, low concentrations of ethanol solutions are toxic to J2 and ethanol concentration of 48% reduces the lipid content of *H. glycines* J2.

Acknowledgements

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