WILLIAN CÉSAR TERRA

COMPOSTOS ORGÂNICOS VOLÁTEIS EMITIDOS POR *FUSARIUM OXYSPORUM* APRESENTAM ATIVIDADE NEMATICIDA

LAVRAS-MG
2016

WILLIAN CÉSAR TERRA

COMPOSTOS ORGÂNICOS VOLÁTEIS EMITIDOS POR *FUSARIUM OXYSPORUM* APRESENTAM ATIVIDADE NEMATICIDA

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós Graduação em Agronomia/Fitopatogia, área de concentração Nematologia, para a obtenção do título de Doutor.

Orientador
PhD. Vicente Paulo Campos

LAVRAS-MG
2016

Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Terra, Willian César.
Compostos orgânicos voláteis emitidos por *Fusarium oxysporum* apresentam atividade nematicida / Willian César Terra. – Lavras: UFLA, 2016.
68 p.: il.

Tese (doutorado)–Universidade Federal de Lavras, 2016.
Orientador(a): Vicente Paulo Campos.
Bibliografia.

1. Volatile Organic Compounds. 2. Root-knot nematodes. 3. *Fusarium oxysporum*. I. Universidade Federal de Lavras. II. Título.

WILLIAN CÉSAR TERRA

COMPOSTOS ORGÂNICOS VOLÁTEIS EMITIDOS POR *FUSARIUM OXYSPORUM* APRESENTAM ATIVIDADE NEMATICIDA

VOLATILES ORGANIC COMPOUNDS EMITTED BY FUSARIUM SHOWS NEMATICIDAL ACTIVITY

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós Graduação em Agronomia/Fitopatogia, área de concentração Nematologia, para a obtenção do título de Doutor.

APROVADA em 12 de setembro de 2016.

PhD. Ludwig H. Pfenning	UFLA
Dr. Marcio Pozzobon Pedroso	UFLA
Dr. Eduardo Souza Freire	UniRV
Dra. Maria Eloisa Salustiano	Epamig

Orientador
PhD. Vicente Paulo Campos

LAVRAS-MG
2016

AGRADECIMENTOS

A Deus, pela vida.

Aos meus pais Júlio César Terra e Tereza Cândida da Silva Terra, por tudo, a minha irmã Amanda Terra e a todos os familiares.

À Universidade Federal de Lavras (UFLA), por me acolher nestes últimos dez anos. Ao CNPq e Fapemig, pela bolsa de estudo.

Ao Prof. Vicente Paulo Campos, pela orientação, compreensão, paciência e por todos os ensinamentos.

Aos membros da banca, Prof. Marcio P. Pedroso, Prof. Ludwig H. Pfenning, Prof. Eduardo S. Freire e Maria E. Salustiano, pela disponibilidade e prontidão em aceitar o convite.

Aos demais professores dos DFP/UFLA, em especial ao prof. Flavio Medeiros. Aos funcionários do Departamento de Fitopatologia, em especial a Elenice e ao Carlos.

Aos amigos da nematologia Eduardo Freire, Júlio Carlos, Lilian, Liliana, Cléber, Tarlei, Aline, Luma, Naldo, Jeanny, Fabiola, pela companhia neste período, pela paciência, apoio.

Aos grandes amigos Samuel Júlio, Alisson Lucrécio, Glauco, Fábio, André, Eugênio, Fabiano, Gustavo, Helon e Fernando.

À minha esposa Marcela, pela convivência e pelo carinho.

RESUMO

Ensaios foram realizados para se entender a atuação dos compostos orgânicos voláteis (COVs) emitidos por *Fusarium oxysporum* isolado 21 (*Fo-21*) sobre os fitonematoides. Através da análise dos compostos por cromatografia gasosa acoplada espectrometria de massas (CG-MS), foram detectados 50 compostos nas emissões de *Fo-21*. As principais classes de compostos emitidos foram sesquiterpenos, ésteres e alcoóis. A água exposta aos COVs de *Fo-21* causou 100% de imobilidade em juvenis de segundo estádio (J_2) de *Meloidogyne incognita*, enquanto a inoculação em tomateiro desses J_2 resultou em redução de até 70% da infectividade e de até 65% da reprodução. A aplicação da água exposta aos COVs em substrato infestado com J_2 de *M. incognita* resultou em 56% de redução no número de galhas e 49% no número de ovos. A atividade nematicida dos oito COVs emitidos em maior abundância por *F.o-21* foi avaliada *in vitro* sobre *M. incognita* e *Heterodera schachtii*. Os compostos acetato de 2-metilbutila (**1**), acetato de 3-metilbutila (**2**), acetato de etila (**7**), acetato de 2 – metilpropila (**8**), na concentração de 500 $\mu g.ml^{-1}$, causaram mortalidades entre 100% e 83,8%, em J_2 de *M. incognita* e *H. schachtii*. A concentração letal (CL_{50}) dos compostos para J_2 de *M. incognita* variou entre 236 e 198 $\mu g.ml^{-1}$, enquanto que nas mesmas condições o nematicida comercial carbofuran (2,3-dihydro-2,2-dimethyl-1-benzofuran-7-yl N-methylcarbamate) foi de 191 $\mu g.ml^{-1}$. Ovos expostos aos compostos **1**, **2**, **7** e **8**, por 72 h, tiveram reduzidas a eclosão do J_2 em 36,5%, 55,2%, 91,0% e 10,3%, e a infectividade de 53%, 53%, 32% e 39%, respectivamente. Quando os compostos foram aplicados em mudas de tomateiro infestadas com *M. incognita* o composto **1** reduziu o número de galhas por grama de raiz em 27,5% quando comparado com o controle negativo sem aplicação de nematicida.

Palavras-chave: Compostos orgânicos voláteis. Nematoides de galhas. *Fusarium oxysporum*.

ABSTRACT
A series of assays was carried out aiming to understand the nematicidal activity of the volatile organic compounds (VOCs) emitted by Fusarium oxysporum isolated 21 (*Fo-21*) on plant-parasitic nematodes. Through analysis by gas chromatography coupled to mass spectrometry (GC-MS) 50 compounds were detected in the *Fo-21* emission. The main classes of compounds emitted by *Fo-21* were sesquiterpenes, esters and alcohols. Water exposed to VOCs caused 100% immobility in second-stage juveniles (J_2) of *Meloidogyne incognita*. In addition, inoculation of those J_2 in tomato decreased infectivity up to 70% and reproduction up to 65%. Application of water exposed to VOCs in substrate infested with *M. incognita* J_2 resulted in 56% reduction in number of galls and 49% in number of eggs. The eight most abundant VOCs emitted by *F.o–21* were tested against *M. incognita* and *Heterodera schachtii*. Compounds 2–methylbutyl acetate **(1)**, 3-methylbutyl acetate **(2)**, ethyl acetate **(7)** and, 2-methylpropyl acetate **(8)**, caused *in vitro* mortality of 100%, 92.5%, 100% and 83.8%, respectively, in *M. incognita* and 90.0%, 88.8%, 96.3% and 97.5% mortality, respectively, on *H. schachtii* J_2, when tested at a concentration of 500 $\mu g.ml^{-1}$. The lethal concentration (LC_{50}) for compounds 1, **2**, **7** and **8**, in *M. incognita* J_2 was of 236, 198, 213 and 218 $\mu g.ml^{-1}$, respectively. Under the same conditions, the commercial nematicide carbofuran (2,3-dihydro-2,2-dimethyl-1-benzofuran-7-yl N-methylcarbamate) showed a LC_{50} of 191 $\mu g.ml^{-1}$. Eggs exposed to compounds **1**, **2**, **7** and **8**, for 72 h showed a reduction in hatching of 36.5%, 55.2%, 91.0% and 10.3%, respectively, and reduction in infectivity of 53.0%, 53.0%, 32.0 % and 39.0%, respectively. When compounds were applied in tomato seedlings infested by *M. incognita,* compound **1** reduced the number of galls per gram of root in 27.5% when compared to the negative control (without the application of nematicide).

Keywords: Volatiles organic compounds. Root-knot nematodes. *Fusarium oxysporum*

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO GERAL

Os fitonematoides constituem fator limitante à agricultura no mundo. A presença deles em áreas agrícolas pode levar a perdas quantitativas e qualitativas, além da redução do valor de venda da propriedade. Dentre os fitonematoides, os nematoides de galhas, *Meloidogyne* spp, representam o principal grupo, causando em torno de 70% das perdas atribuídas aos fitonematoides (SASSER; FRECKMAN, 1987).

Diversas são as estratégias para o manejo da população de fitonematoides a níveis inferiores ao limiar de dano econômico (OKA, 2010). Contudo, o manejo no campo, desses micro-organismos, é tarefa árdua. E em muitos casos os nematicidas constituem a opção mais utilizada pelos produtores/extensionistas. Os nematicidas apresentam como vantagens em relação às outras opções de controle a sua eficiência aliada a resultados rápidos. Entretanto, os principais nematicidas foram retirados do comércio nos últimos anos (MCCARTER, 2008). Assim, o número de moléculas comerciais com atividade nematicida encontra-se restrito, fato que tem motivado a busca por novas moléculas comerciais.

Na última década, estudos têm sugerido que compostos orgânicos voláteis (COVs) apresentam potencial para serem desenvolvidos como nematicidas comerciais (CAMPOS; PINHO; FREIRE, 2010; COSTA et al., 2015; GRIMME et al., 2007; GU et al., 2007). Os COVs caracterizam-se por terem até 20 átomos de carbono, alta pressão de vapor e um caráter lipofílico (EFFMERT et al., 2012). Uma rica fonte de emissão de COVs são os micro-organismos habitantes de solo. Em torno de 1000 COVs emitidos por 480 espécies de fungos e

bactérias estão descritos e reunidos em uma base de dados http://bioinformatics.charite.de/mvoc/ (LEMFACK et al., 2015).

Fusarium oxysporum é a espécie fúngica encontrada em maior frequência e abundância em massas de ovos de *Meloidogyne* spp (COSTA et al., 2015; FREIRE et al., 2012; TRIFONOVA; KARADJOVA; GEORGIEVA, 2009). Isso talvez decorra da utilização das massas de ovos na nutrição do fungo. No entanto, isolados de *F. oxysporum* obtidos de massas de ovos de *M. exigua* são capazes de emitir COVs com atividade nematicida, sendo que o isolado denominado de ***Fo-21*** por Freire *et al*., 2012*,* emite COVs com atividade nematicida próximas de 100 %.

Muitas são as lacunas no entendimento do processo que leva à intoxicação dos fitonematoides quando expostos aos COVs emitidos por *F. oxysporum*. A solubilização na água dos COVs emitidos por micro-organismos pode explicar, em parte, a distribuição destes no solo e o processo de exposição dos fitonematoides a eles (BARROS et al., 2014). Assim, o papel da água como veículo de solubilização e retenção de COVs foi explorado. No presente trabalho, buscou-se identificar os COVS emitidos por ***Fo-21*****,** assim como aqueles solubilizados na água exposta aos COVs de ***Fo-21***. O. A identificação dos COVs emitidos pelo fungo foi feita através da análise dos compostos por cromatografia gasosa acoplada à espectrometria de massas (CG-MS) realizada na Central de Análise e Prospecção Química (CAPQ), departamento de Química da Universidade Federal de Lavras. Para comprovar "*in vivo"* o efeito da água exposta aos COVs na infectividade e reprodução de *M. incognita* foi apresentada uma adaptação na metodologia da placa de polipropileno bipartida (FERNANDO et al., 2005). Dando

prosseguimento a tal estudo, foi investigada a atividade nematicida individual dos oito COVs encontrados em maior abundância nas emissões de *F.o-21*, buscando-se assim avaliar o potencial de tais compostos para o desenvolvimento de nematicida comercial. Para tanto, avaliou-se: (1) a atividade nematicida *in vitro* dos COVs, em concentração única, sobre J_2 de *M. incognita* e *Heterodera schachtii* (2) as concentrações letais para 50% (CL_{50}) dos J_2 de *M. incognita*; (3) as atividades dos COVs sobre a eclosão dos J_2 de *M. incognita*; (4) a infectividade em tomateiro, dos J_2 provenientes de ovos expostos aos COVs; e (5) a infectividade em tomateiro dos J_2 de *M. incógnita,* após aplicação dos compostos diretamente em substrato infestado.

2 REFERENCIAL TEÓRICO

2.1 Os fitonematoides e sua importância econômica

Os fitonematoides representam um grande obstáculo ao desenvolvimento da agricultura no mundo. McCarter (2008), atribui a esses micro-organismos redução de aproximadamente 10% da produção mundial de alimentos. Os fitonematoides apesar da sua importância são pouco conhecidos pelos agricultores. As justificativas para tal fato advêm do tamanho microscópio, normalmente de 0,2 a 4,0 mm, e pela preferência ao parasitismo de órgãos vegetais subterrâneos. Assim, alguns nematologistas denominam os fitonematoides como “os inimigos invisíveis das plantas” (FERRAZ; BROWN, 2016). A junção do potencial danoso dos fitonematoides com o pouco conhecimento deles pelos agricultores/extensionistas tem criado um ambiente favorável a sua ampla disseminação nas regiões agrícolas brasileiras (CASTRO et al., 2008; SILVA et al., 2004; SILVA; OLIVEIRA, 2010).

Entre os fitonematoides, os nematoides de galhas, *Meloidogyne* spp, destacam-se pelas perdas econômicas e pelo sofisticado modelo de parasitismo. Sasser e Freckman (1987), atribuem aos nematoides de galhas 70% dos danos causados pelos fitonematoides. O modelo de parasitismo dos nematoides de galhas é tido como o mais sofisticado dentre todos os fitonematoides (GOVERSE; SMANT, 2014). O processo de parasitismo que se inicia na penetração das raízes, passando pela migração intercelular e pela formação e manutenção do sítio de alimentação envolve a síntese nas glândulas esofagianas e a liberação, através do estilete de diversas enzimas (endoglucanases, xilanases, galacturonases, pectato liases, poligalacturonases) e centenas de efetores (ESCOBAR et al., 2015). E entre os nematoides de galhas, uma espécie é digna de destaque. *Meloidogyne incognita* pode ser considerado o patógeno de plantas que mais causa danos a agricultura. Tal afirmação se alicerça na ampla distribuição geográfica dessa espécie aliada à capacidade de parasitar a maioria das espécies vegetais cultiváveis (TRUDGILL; BLOCK, 2001).

2.2 O panorama de moléculas com atividade nematicida e potencial comercial

A revolução verde iniciada em meados do século XX tornou grande parte da agricultura dependente de moléculas de origem sintética. Atualmente, as altas produtividades alcançadas no campo têm alicerce na utilização massiva dos agroquímicos (fertilizantes sintéticos, inseticidas, fungicidas, herbicidas, nematicidas etc). Muitos foram os benefícios trazidos com a utilização dos referidos agroquímicos, pois foi através da utilização desses produtos que a agricultura conseguiu alimentar uma população mundial que, durante o século XX, passou de

1,5 bilhões de pessoas para 6,1 bilhões (ORTIZ-OSPINA; ROSER, 2016).

Contudo, aliado aos benefícios advindos da utilização dos agroquímicos, vieram os malefícios. Já na década de 1960, um importante livro "Silent Spring", escrito por Rachel Carson, advertia sobre a perda da diversidade de pássaros a partir da utilização maciça dos inseticidas nos Estados Unidos.

A fitonematologia sofreu influência da revolução verde, pois nas décadas de 1940 e 1950 foi descoberta a possibilidade do controle dos fitonematoides, através da utilização dos nematicidas e, provou-se que o controle desses micro-organismos levava a ganhos substanciais de produtividade (TAYLOR, 2003). Tal descoberta também contribuiu para o crescimento da fitonematologia como uma disciplina acadêmica (MCCARTER, 2008).

A partir da sua descoberta, os nematicidas se tornaram uma das principais ferramentas utilizadas no manejo dos fitonematoides. Os primeiros nematicidas dividiam-se em duas classes: os fumigantes (DBCP, chloropicrin, 1,3-D, DD mixture, EDB, MBr) e os não fumigantes (Aldicarb, Carbofuran, Ethoprop, Fenamiphos, Fensulfothion, Oxamyl), grande maioria deles incluídos entre carbamatos e organofosforados (TAYLOR, 2003). Todos esses nematicidas compartilhavam uma característica importante, todos apresentavam uma ampla atividade biocida. No ano 1977, nos Estados Unidos, a produção do DBCP (NEMAGON e FUMAZONE) foi interrompida, por causar esterilidade no homem. Logo depois a maioria dos compostos fumigantes foi retirada do mercado, pois, além da alta toxicidade, contribuíam para o aumento do buraco na camada de ozônio.

Já os nematicidas não fumigantes, principalmente os carbamatos e organofosforados, permaneceram no comércio por um período maior, inclusive ainda hoje são as principais opções encontradas pelos produtores. Contudo, alguns dos principais nematicidas não fumigantes também tiveram sua produção interrompida, primeiro nos países desenvolvidos e depois nos países em desenvolvimento. Um exemplo típico foi o caso do nematicida Aldicarb (Temik®), que foi proibido no Brasil em 2012.

Nas últimas décadas, a restrição ao uso dos principais nematicidas, aliado a interrupção da fabricação de outros, resultou em uma lacuna no mercado de moléculas com atividade nematicida e potencial comercial. Com exceção do progressivo uso da abamectina no tratamento de sementes, nenhuma classe de nematicidas eficientes foi lançada comercialmente, desde a década de 1970 (MCCARTER, 2008). Nos Estados Unidos, em março de 2015, um novo nematicida Nimitz® advindo de uma nova classe química (Fluoro-alkenyle) recebeu o registro junto a United States Environmental Protection Agency (EPA). No Brasil, esse produto se encontra em fase de registro.

2.3 Fungos como emissores de compostos orgânicos voláteis

Os fungos são emissores de um grande número de COVs (BITAS et al., 2015; BUSKO et al., 2014). A diversidade e a quantidade de COVs emitidos por uma determinada espécie fúngica são condicionadas a fatores ambientais como temperatura, luminosidade e, principalmente, o substrato de cultivo do fungo (EZRA; STROBEL, 2013). A ciência utiliza os COVs emitidos por fungos para várias finalidades. A seguir e de forma resumida são citadas algumas das utilidades dos COVs emitidos por fungos: (1) **Olfato e aroma**, voláteis

fúngicos contribuem para propriedades desejáveis de certos queijos, salsichas e cervejas (BRUNA et al., 2001; KINDERLERER, 1989); (2) **Indicadores de deterioração**, em edifícios as emissões de COVs permitem a identificação de fungos causadores de mofos (MATYSIK; HERBARTH; MUELLER, 2008); (3) **Quimiotaxonomia**, 27 isolados de *Fusarium graminearum* foram agrupados em três grupos de acordo com as das similaridades nas emissões de COVs (BUSKO et al., 2014); (4) **Indução de resistência**, os voláteis emitidos pelos fungos emitidos pelos fungos *Ampelomyces* sp. e *Cladosporium* sp. reduziram a severidade da doença causada por *Pseudomonas syringae* pv. *tomato* em *Arabidopsis thaliana* (NAZNIN et al., 2014); (5) **Promoção de crescimento**, isolados de *Fusarium oxysporum* promovem o crescimento em *A.thaliana* (BITAS et al., 2015); (6) **Interação com outros micro-organismos**, vários são os estudos revelando o papel dos voláteis emitidos pelos fungos afetando outros micro-organismos (HUNG; LEE; BENNETT, 2015). *Muscodor albus*, um fungo endofítico, originalmente isolado de árvores de canela, é um bom exemplo. Os COVs emitidos por esse fungo são capazes de matar uma série de patógenos vegetais (fungos, bactérias e nematoides) (GRIMME et al., 2007; STROBEL et al., 2001); (7) **Diagnóstico de doenças**, em muitos países do continente africano, o diagnóstico da tuberculose é feito através da detecção por ratos dos COVs emitidos por *Mycobacterium tuberculosis,* agente causal da maioria das tuberculoses (MGODE et al., 2012).

Por que os fungos emitem compostos orgâncos voláteis? Na bela revisão feita por Hung, Lee e Bennett (2015), os autores citam um aforismo utilizado pelo geneticista Theodosius Dobzhansky “nada na

biologia faz sentido excepto à luz da evolução", para responder a questão levantada. Os COVs emitidos por fungos certamente estão envolvidos em razões adaptativas, para facilitar a comunicação com o meio ambiente, para ajudar na reprodução, para atrair ou repelir outros organismos e para muitos outros papéis funcionais (HUNG; LEE; BENNETT, 2015). Entender a relação entre os COVs e os micro-organimos pode levar à criação de novos métodos de controle.

2.4 A bioprospecção de moléculas com atividade nematicida a partir do conjunto de COVs emitidos por fungos

A partir do aumento dos estudos sobre as emissões de COVs por espécies fúngicas e bacterianas, ocorrido principalmente na última década, um corpo de evidências tem indicado que os voláteis podem fornecer moléculas alternativas aos atuais compostos utilizados na agricultura (HUNG; LEE; BENNETT, 2015). Em torno de 1000 COVs emitidos por 480 espécies de fungos e bactérias estão descritos e reunidos em uma base de dados http://bioinformatics.charite.de/mvoc/ (LEMFACK et al., 2015).

Especificamente em relação aos COVs emitidos pelos fungos e com atividade nematicida, poucos são os trabalhos encontrados na literatura, ao menos no nosso conhecimento. Grimme et al. (2007), demonstraram a atividade nematicida dos COVs emitidos por *M. albus* sobre J_2 de *M. incognita*. Yang et al. (2012), relatam a atividade nematicida dos voláteis emitidos por *Trichoderma* sp. a *Bursaphelenchus xylophilus*. A atividade nematicida dos COVs emitidos por diversos isolados de *Fusarium oxysporum* sobre *M. incognita* foi demonstrada por Costa et al. (2015) e Freire et al. (2012).

A bioprospecção de COVs com atividade nematicida, que se constitui na busca sistemática por voláteis provenientes de seres vivos, com potencial econômico e, eventualmente, potencial para o desenvolvimento de um produto, tem sido estimulada a partir da comprovação de que fungos emitem COVs letais aos fitonematoides. Kanchiswamy, Malnoy e Maffei (2015), sugerem que após o considerável progresso que já foi feito nos últimos anos, no entendimento dos diversos papeis desempenhados pelos COVs emitidos por micro-organismos, chegou a hora dos estudos partirem para a experimentação em casa de vegetação e no campo. Outro passo importante na pesquisa com COVs seria avaliar a atuação individual de cada composto sobre o alvo biológico.

3 REFERÊNCIAS

BARROS, A. F. et al. Nematicidal activity of volatile organic compounds emitted by *Brassica jucea, Azadirachta indica, Canavalia ensiformis, Mucuna pruriens* and *Cajanus cajan* against *Meloidogyne incognita.* **Applied Soil Ecology**, Florença, v. 80, p. 34-43, Aug. 2014.

BITAS, V. et al. *Fusarium oxysporum* volatiles enhance plant growth via affecting auxin transportand signaling. **Frontiers in Microbiology**, New York, v. 6, n. 6, p. 1248, Nov. 2015.

BRUNA, J. M. et al. The contribution of *Penicillium aurantiogriseum* to the volatiles composition and sensory quality of dry fermented sausages. **Meat Science**, Cowra, v. 59, n. 1, p. 97–107, Feb. 2001.

BUSKO, M. et al. Quantitative volatile compound profiles in fungal cultures of three different *Fusarium graminearum* chemotypes. **FEMS Microbiol. Letter,** Malden, v. 359, n. 1, p. 85-93, Oct. 2014.

CAMPOS, V. P.; PINHO, R. S. C.; FREIRE, E. S. Volatiles produced by interacting microorganisms potentially useful for the control of plant pathogens. **Ciência e Agrotecnologia**, Lavras, v. 3, n. 3, p. 525-535, maio/jun. 2010.

CASTRO, J. M. C. et al. Levantamento de fitonematoides em cafezais do Sul de Minas Gerais. **Nematologia Brasileira**, Piracibaca, v. 32, n. 1, p. 56-64, mar. 2008.

COSTA, L. S. A. S. et al. Microbiota from *Meloidogyne exigua* egg masses and evidence for the effect of volatiles on infective juvenile survival. **Nematology**, Leiden, v. 6, n. 6, p. 715-724, 2015.

EFFMERT, U. et al. Volatile mediated interactions between bacteria and fungi in the soil. **Journal of Chemical Ecology,** Tampa, v. 38, n. 6, p. 665-703, June 2012.

ESCOBAR, C. et al. Overview of Root-Knot nematodes and giant cells. **Advances in Botanical Research**, v. 73, n. 1, p. 1-32, Mar. 2015.

EZRA, D.; STROBEL, G. A. Effect of substrate on the bioactivity of volatile antimicrobials produced by *Muscodor albus.* **Plant Science**, Philadelphia, v. 165, n. 6, p. 1229-1238, Dec. 2013.

FERNADO, W. G. Identification and use of potential bacterial organic antifungal volatiles in biocontrol. **Soil Biology and Biochemistry**, Leicestershire, v. 5, n. 37, p. 955-964, May 2005.

FERRAZ, L. C. C. B.; BROWN, D. J. F. **Nematologia de plantas:** fundamentos e importância. Campos dos Goytacazes: Sociedade Brasileira de Nematologia, 2016. 251 p.

FREIRE, E. S. et al. Volatile substances on the antagonism between fungi, bacteria and *Meloidogyne incognita* and potentially fungi for nematode control. **Journal of Nematology**, Beltsville, v. 4, n. 4, p. 321-328, Dec. 2012.

GOVERSE, A.; SMANT, G. The activation and suppression of plant innate immunity by parasitic nematodes. **Annual Review of Phytopathology**, Palo Alto, v. 52, p. 243-265, May 2014.

GRIMME, E. et al. Comparison of *muscodor albus* volatiles with a biorational mixture for control of seedling diseases of sugar beet and

root-knot nematode on tomato. **Plant Disease**, Saint Paul, v. 91, n. 2, p. 220-225, Feb. 2007.

GU, Y. I. et al. Evaluation and identification of potential organic nematicidal volatiles from soil bacteria. **Soil Biology & Biochemistry**, Leicestershire, v. 39, n. 10, p. 2567–2575, Oct. 2007.

HUNG, R.; LEE, S.; BENNETT, J. W. Fungal volatile organic compounds and their role in ecosystems. **Applied Microbiology and Biotechnology**, Heidelberg, v. 99, n. 8, p. 3395-3405, Apr. 2015.

KANCHISWAMY, C. N.; MALNOY, M.; MAFFEI, M. E. Chemical diversity of microbial volatiles and their potential for plant growth and productivity. **Frontiers in Plant Science**, Lausanne, v. 6, p. 1-23, Mar. 2015.

KINDERLERER, J. L. Volatile metabolites of filamentous fungi and their role in food flavor. **J Appl Bacteriol Symposium Supplement**, p. 133–144, Sept. 1989.

LEMFACK, M. C. et al. mVOC: a database of microbial volatiles. **Nucleic Acids Research**, Oxford, v. 42, n. 1, p. 744–748, Dec. 2015.

MATYSIK, S.; HERBARTH, O.; MUELLER, A. Determination of volatile metabolites originating from mould growth on wall paper and synthetic media. **Journal of Microbiological Methods,** Guelph, v. 75, n. 2, p. 182-187, Oct. 2008.

MCCARTER, J. P. Molecular approaches toward resistance to plant-parasitic nematodes. In: BERG, R. H.; TAYLOR, C. G. (Ed.). **Cell biology of plant nematode parasitic**. Berlin: Plant Cell Monographs, 2008. p. 239-267.

MGODE, G. F. et al. Mycobacterium tuberculosis volatiles for diagnosis of tuberculosis by Cricetomys rats. **Tuberculosis**, New South Wales, v. 92, n. 6, p. 535-542, Nov. 2012.

mVOC. **Microbial volatile organic compound database**. Berlin: Institute for Biosciences Biochemistry, 2016. Disponível em: <http://bioinformatics.charite.de/mvoc/>. Acesso em: 28 jun. 2016

NAZNIN, H. A. et al. Systemic resistance induced by volatile organic compounds emitted by plant growth-promoting fungi in *Arabidopsis thaliana*. **PLoS One**, San Francisco, v. 1, n. 9, p. e86882, Jan. 2014.

OKA, Y. Mechanisms of nematode suppression by organic soil amendments: a review. **Applied Soil Ecology**, Columbus, v. 44, n. 2, p. 101-115, Feb. 2010.

ORTIZ-OSPINA, E.; ROSER, M. World population growth. ***OurWorldInData.org****, Oxford, 2016.* Disponível em: <https://ourworldindata.org/world-population-growth/#note-1>. Acesso em: 15 jul. 2016

SASSER, J. N.; FRECKMAN, D. W. A world perspective on nematology the role ofthe society. In: VEECH, J. A.; DICKSON, D. W. (Ed.). **Vistas on nematology**. Maryland: Society of Nematologists, 1987. p. 7–14.

SILVA, R. A. et al. Ocorrência de *Pratylenchus brachyurus* e *Meloidogyne incognita* na cultura do algodoeiro no estado do Mato Grosso. **Fitopatologia Brasileira**, Viçosa, v. 3, n. 3, p. 337, maio/jun. 2004.

SILVA, R. V.; OLIVEIRA, R. D. L. Ocorrência de *Meloidogyne enterolobii* (Sin. *M. mayaguensis*) em goiabeiras no estado de Minas Gerais, Brasil. **Nematologia Brasileira,** Campos dos Goytacazes, v. 34, n. 3, p. 172-177, set. 2010.

STROBEL, G. A. et al. Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. **Microbiology**, London, v. 147, n. 1, p. 2943–2950, Nov. 2001.

TAYLOR, A. L. Nematocides and nematicides. **Nematropica**, Gainesville, v. 33, n. 2, p. 225-232, Dec. 2003.

TRIFONOVA, Z.; KARADJOVA, J.; GEORGIEVA, T. Fungal parasites of the root-knot nematodes *Meloidogyne* spp. in southern Bulgaria. **Estonian Journal of Ecology**, Tallinn, v. 58, n. 1, p. 47-52, Mar. 2009.

TRUDGILL, D. L.; BLOCK, V. C. Apomitic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. **Annual Review of Phytopathology**, Palo Alto, v. 39, p. 53-77, Sept. 2001.

YANG, Z. et al. Nematicidal effect of volatiles produced by *Trichoderma* sp. **Journal of Asia-Pacific Entomology**, Seoul, v. 15, n. 4, p. 647-650, Dec. 2012.

SEGUNDA PARTE – ARTIGOS

ARTIGO 1

Volatile molecules of *Fusarium oxysporum* are retained in water and control *Meloidogyne incognita*

Willian César Terra, Vicente Paulo Campos, Márcio Pozzobon Pedroso, Alisson Lucrécio da Costa, Eduardo Souza Freire, Iohana Pinto de Pinto, Júlio Carlos Pereira da Silva, Liliana Estupiñan Lopez.

Preparado de acordo com as normas do periódico Biological Control

Abstract

Dissolution in water of volatile organic compounds (VOCs) emitted by rhizospheric flora may explain in part the distribution of VOCs throughout the soil, and thus the process of exposure of plant-parasitic nematodes (PPN) to these molecules. Water was exposed for short periods (0.5 to 24 h) to a gas chamber formed by VOCs emitted by *Fusarium oxysporum* isolate 21 (*Fo-21*). Water exposed to VOCs caused 100% immobility in second-stage juveniles (J_2) of *Meloidogyne incognita*. In addition, inoculation of those J_2 in tomato decreased infectivity up to 70% and reproduction up to 65%. Application of water exposed to VOCs in substrate infested with *M. incognita* J_2 resulted in 56% reduction in number of galls and 49% in number of eggs. This is the first experimental demonstration *in vivo* of toxicity of water exposed to fungal VOCs to a PPN. Through analysis by gas chromatography coupled to mass spectrometry (GC-MS) 28 compounds were identified in the water exposed to the VOCs and 36 compounds were identified in the emission of the fungus. The main classes of compounds emitted by *Fo-21* were sesquiterpenes, esters and alcohols. This is also the first

evaluation of volatile compounds retained in water exposed to fungal VOCs.

Keywords: volatile organic compounds, root-knot nematodes, gas chromatography, *Fusarium oxysporum*

1 INTRODUCTION

In nature, volatile organic compounds (VOCs) are responsible for inter and intraspecific communication and co-participation in numerous antagonistic, mutualistic, and symbiotic interactions (Effmert *et al*., 2012; Kanchiswamy *et al.*, 2015). A rich source of production and emission of VOCs are microorganisms living in soil (Leff & Fierer 2008; Insam & Seewald 2010; Lemfack *et al*., 2014) and in other ecosystems (Campos *et al*., 2010). Around 1000 VOCs emitted by 480 species of bacteria and fungi are described and assembled in the database http://bioinformatics.charite.de/mvoc/; Lemfack *et al*., 2014).

Plant-parasitic nematodes (PPN) are responsible for reduction of approximately 10% of world food production (McCarter, 2008). Among them, nematodes of genus *Meloidogyne* (root-knot nematodes) are responsible for major losses. The species *Meloidogyne incognita* is the plant pathogen causing more damage to agriculture (Trudgill & Block, 2001).

Several management tactics can be used to control nematodes, and commercial nematicides are outstanding for their efficiency (Sikora *et al*., 2005). However, due to their extremely damaging potential to the environment (Oka, 2010) and humans, these products have been withdrawn from the market, thereby reducing control options (Noling, 2002). Except for progressive use of abamectin in seed treatment, no class of efficient nematicides has been commercially launched since the

1970s (McCarter, 2008). The possibility of finding molecules with potential use in agriculture, among the thousands of VOCs produced by microorganisms, has attracted the interest of researchers from various fields (Kanchiswamy *et al.*, 2015).

Plenty of VOCs with nematicidal activity exist and are an alternative strategy for discovering molecules or chemical skeletons that can be transformed into efficient commercial nematicides (Li *et al.,* 2012). VOCs emitted by fungi and bacteria have shown toxicity to PPN (Gu *et al*., 2007; Grimme *et al*., 2007; Freire *et al*., 2012; Costa *et al*., 2015). Specific terminologies have been developed in this area, such as mycofumigation, which refers to the use of volatiles emitted by fungi for controlling microorganisms causing diseases in plants (Strobel *et al.,* 2001). The fungus *Muscodor albus* is the best studied mycofumigation agent, as its VOCs caused growth inhibition of various plant pathogens (Strobel *et al.,* 2001, Grimme *et al.,* 2007, Riga *et al*., 2008, Banerjee *et al*., 2014). The Marrone Bio innovations® company submitted its patent application (US 20140086879 A1) to the United States Environmental Protection Agency (EPA) for registering *M. albus* as a biofumigant in the control of PPN, fungi and insects. AgraQuest, a biopesticide company acquired by multinational Bayer®, have been explored the use of *M. albus* in various agricultural applications. In particular, the company intends to use *M. albus* and its volatiles for soil sterilization, replacing the use of methyl bromide, a highly toxic pesticide and detrimental to ozone layer (Alpha *et al.,* 2015), which has been withdrawn from the market in many countries.

Fusarium oxysporum is a normal component of fungal communities in the rhizosphere and soil, despite containing

phytopathogenic lineages (Gordon & Martyn, 1997). This fungus is often isolated from egg mass of *Meloidogyne spp.* (Trifonova *et al*., 2009, Freire *et al*., 2012, Costa *et al*., 2015). Some isolates of *F. oxysporum* emit VOCs toxic to *M. incognita*, and the exposure of second-stage juveniles (J_2) to these volatiles resulted in fewer galls in tomato plants (*Solanum esculentum*) (Freire *et al*., 2012, Costa *et al*., 2015). In particular, the isolate *F. oxysporum 21 (F.o-21)* emitted VOCs toxic to *M. incognita* J_2 (Freire *et al*., 2012).

Both soil moisture and temperature are reported to affect physical VOCs process in soil. Most VOCs increases their soil uptake and retention at higher soil moisture (Asensio *et al*., 2007). Wheathey (2002) suggests that water favors the action of VOCs on targets near the emission point, as mobility of small molecules (volatiles) by water (dissolution) facilitates VOCs distribution in soil, facilitating their performance close to the production source. Barros *et al*., (2014a) suggest that solubilization of volatile molecules and their retention in water for determined periods prevent losses to the atmosphere, thus increasing their chances of finding and poisoning PPN. Retention or solubility of VOCs in water is dependent on Henry's Law (S = K. P), in which the amount of VOCs retained in water depends on medium pressure and polarity of molecules (Hoff *et al*., 1993).

The effect of application of water exposed to VOCs of *F. oxysporum* in soil infested with PPN is still unknown, as well as the composition of molecules retained in water. Dissolution of VOCs emitted by *F. oxysporum* in water may explain, in part, the fungus's interference in rhizosphere balance in its search for controlling PPN. The aim of this work was to prove *in vitro* and *in vivo* the effect of water

exposed to VOCs of *F.o-21* on mobility, infectivity and reproduction of *M. incognita*. In addition, the profile of VOCs emitted by *Fo-21* and the compounds retained in water were identified through gas chromatography coupled with mass spectrometry (GC-MS).

2. MATERIAL AND METHODS

2.1 Collecting eggs and second-stage juveniles of *Meloidogyne incognita*

Pure populations of *Meloidogyne incognita* were multiplied in tomato plants and maintained in a greenhouse for three months. Galled roots were separated from soil, washed in still water, and cut into pieces 0.5 cm long segments. Eggs were extracted from roots by the process described by Hussey & Barker (1973). The eggs were placed in a hatching chamber. Second-stage juveniles (J_2) hatched between the second and third day were used in assays.

2.2 Cultures of *Fusarium oxysporum*

The isolate *F.o-21* obtained from *Meloidogyne exigua* egg's mass was selected for the tests due to its ability to emit VOCs toxic to *M. incognita* (Freire *et al*. 2012). The isolate is deposited under the number (CML 3605) in the Coleção Micologica de Lavras, Department of Plant Pathology - Federal University of Lavras, Brazil, preserved in water (Castallani's method). The isolate was subcultured in malt culture medium (MA) (malt 20 $g.L^{-1}$ agar 20 g. L^{-1}), and incubated at 25^0C for six days. A 10 mm plug was taken from the border of the colonies and subcultured to new plates with MA medium. Then, they were kept at 25^0C until use.

2.3 Toxicity of water after exposure to volatile organic compounds from *Fusarium oxysporum* isolate 21

We used the two-compartmented Petri dishes (100 mm x 15 mm) made of plastic that contained a center partition (I-plates) Fernando *et al*. (2005) with modification. In the surface of a plate cover (opposite side to the fungus culture), a perforation of 2 mm diameter was made, enough to allow the insertion of a hypodermic needle (5 ml), which was sealed using adhesive tape Adelbras®. The hole was used to add water into the I-plates without removing the covers and consequently losing the VOCs produced during the first days of fungal growth. The substrate used for fungal growth was the medium yeast extract sucrose agar (YES) (yeast extract 20 $g.L^{-1}$, sucrose 150 $g.L^{-1}$, $MgSO_4.7H_2O$ 0.5 $g.L^{-1}$, agar 20 $g.L^{-1}$), as recommended for analysis of secondary metabolites by the Centraal Bureau voor Schimmelcultures (CBS) Fungal Biodiversity Center (Busko *et al*., 2014). One half of the I-plates were filled with YES medium, where a 10 mm plug of *F.o-21* was replicated. Then, plates were closed and sealed with plastic film Parafilm®. As control, I-plates with YES medium without fungus were used. Plates were incubated at 28^0 C in the darkness. After incubation for five or seven days (independent assays), 2 ml of deionized water were injected in the compartment adjacent to the fungal culture. After removal of the needle, the hole was re-sealed with adhesive tape. Thus, water was introduced into a gas chamber containing volatiles emitted by *F.o-21* during the first five or seven days of incubation. The exposure time to the gas chamber was 30 min, 1 h, 2 h, 3 h, 6 h, 12 h or 24 h.

After each exposure time, the toxicity of water to *M. incognita* J_2 was evaluated. For this purpose, 1 ml of water exposed to VOCs was

poured in a 2 ml screw top microtube pre-filled with an aqueous suspension of 1 ml containing 600 J_2. The microtube was closed and stored for 24 h in an incubator at 28^0 C. Then, an aliquot (150 µl) containing approximately 45 J_2 was collected and transferred to ELISA polypropylene microplate, and both the mobile and immobile J_2 were quantified. After that, the microplate was left unsealed on the laboratory bench at room temperature. After 24 h, the number of mobile and immobile J_2 was counted again, and immobile J_2 were considered dead.

2.4 Infectivity and reproduction of *Meloidogyne incognita* in tomato after exposure of second-stage juveniles to volatiles of *F.o-21* retained in water

Using the same methodology described in the previous assay, 2 ml of deionized water were exposed for 1 h, 2 h, 3 h, 6 h, 12 h or 24 h to VOCs of *F.o-21* in closed gas chamber - seven days of fungal growth. After its exposure to VOCs, water (1 ml) was removed from the I-plate and placed in a 2 ml screw top microtube to which an aqueous suspension of 1 ml containing 450 J_2 was added. The microtube was closed and stored in incubator at 28^0 C. After 24 h, the microtube was opened and with the aid of a pipette the J_2 were equally distributed in four holes around the stems of tomato (*Solanum lycopersicum* L. cv. Santa Clara). All inoculated seedlings were 30 days old at the time of the inoculation. Seedlings were grown in Styrofoam trays with 72 cells filled with Plantmax® substrate. Carbofuran nematicide (2,3dihydro-2,2-dimethyl-7-benzofuranyl methyl carbamate), Aldrich 98% at concentration 400 $\mu g.ml^{-1}$ H_2O was used as positive control, while the negative controls were (1) water exposed for 12 h to gases of YES medium and (2) distilled water. Foliar fertilization was performed on

plants at seven-day intervals, using Biofert® foliar fertilizer. The plants were kept in the greenhouse. The number of galls and eggs per gram of root was quantified 35 days after the inoculation. Eggs were extracted by the Hussey & Barker technique, 1973, and counted with a microscope.

2.5 Application of water exposed to volatiles directly in tomato plants inoculated with second stage juveniles of *Meloidogyne incognita*

Using the same methodology described in 2.3, water was exposed for 12 h to VOCs of *F.o-21* in a gas chamber - seven days of fungal growth. Tomato seedlings cultivar Santa Clara® grown in plastic cups 50 cm^3 filled with Plantmax® substrate were infested with an aqueous suspension of 2 ml containing 300 J_2. All inoculated seedlings had been sowed 30 days before. The J_2 were placed in four holes around the stems of seedlings. Then, 4 ml of water exposed to VOCs were applied to each seedling, with 2 ml applied at the time of substrate infestation with J_2 and 2 ml after 24 h. As negative control water exposed to VOCs of YES medium was used. The plants were kept in the greenhouse. The number of galls and eggs per gram of root was quantified 35 days after the inoculation. Eggs were extracted by the Hussey & Barker technique, 1973, and counted with a microscope.

2.6 Identification of volatile compounds emitted by *Fusarium oxysporum* and retained in water by GC-MS

Water was exposed for 30 min, 1 h, 2 h, 3 h, 6 h, 12 h or 24 h to VOCs of *F.o-21* in closed chamber with 7 days of fungal growth, as described before. As control, water was also exposed to VOCs emitted by YES medium without *F.o-21*. Then, water was transferred to SUPELCO™ SPME tubes (Sigma-Aldrich, Bellefonte, PA, USA) 80 x

28 mm with screw cap and inner top layer of silicone, which ensures complete sealing. It was also analyzed the VOCs emitted by *F.o-21* in the air of the gas chamber. Therefore, 8 ml of YES medium were transferred to SUPELCO^{TM} tubes where a plug of *F.o-21* was replicated. The tubes were left in incubator at 28^0C for seven days.

Volatile identification was conducted at the Center for Analysis and Chemical Prospecting (Department of Chemistry/UFLA). VOCs were extracted via headspace solid-phase micro extraction (SPME) (Arthur & Pawliszyn, 1990). The following parameters were adopted: DVB/CAR/PDMS (Divinylbenzene, Carboxen, and Polydimethylsiloxane) fiber, temperature and extraction time were 35°C and 30 min., respectively. A GC-MS QP 2010 Ultra (Shimadzu, Japan) gas chromatograph coupled to a mass spectrometer equipped with a AOC-5000 (Shimadzu, Japan) automatic injector for liquids and gas and an HP-5 (5% phenyl-95% dimethyl siloxane) 30 m x 0.25 mm × 0.25 μm column was used to separate the VOCs. The injector, interface and ion detector temperatures were 250°C, 240°C and 200°C. The injector operated in the splitless mode or split mode 1:2 based on the sample peak intensity. The carrier gas was grade 5.0 helium with a flow of 1.0 ml.min^{-1}. The GC oven temperature was increase at a rate of 3°C min^{-1} from 40° C to 130° C and then at 10° C min^{-1} to 240° C. The MS scan range was set from 40 to 350 A.M.U. To identify the VOCs, the mass spectrum of each peak was obtained via the Automated Mass Spectral Deconvolution and Identification System (AMDIS) v. 2.63 software. The VOCs were identified by comparing the mass spectra to those in the NIST library using the Mass Spectral Search Programv. 1.7 (NIST, Washington DC, USA) software and the experimentally obtained

retention indexes (RI Exp.) to those reported in the liter-ature (RI Lit.) (Adams, 2007; NIST, 2013; Rohloff & Bones, 2005). For the mass spectra comparison, only spectra with a similarity better than 80% were considered. The experimental retention indices were obtained by injecting a homologous series of alkanes.

2.7 Statistical analysis

All of the assays were repeated using a completely randomized experimental design with six or seven replicates per treatment. Results were previously subjected to analysis of normality (Shapiro-Wilk) and homogeneity of error variance (Bartlett). When these assumptions were met, an ANOVA (F test) was performed. In the first experiment, the relationship between percentage of J_2 immobility and water exposure time (hours) to the gas chamber was analyzed using generalized linear models (GLMS). In the second experiment, the means were compared by Scott-Knot test ($P < 0.05$).

3 RESULTS

3.1 Toxicity of water after exposure to volatile organic compounds of *Fusarium oxysporum* isolate 21

Water exposed to both gas chambers (5 or 7 days) became toxic to *M. incognita* J_2, causing immobility. In the two independent assay (1A and 1B), from 1h, water exposed to the gas chamber with 7 days caused 100% immobility to *M. incognita* J_2 (Figure 1). In the gas chamber where the fungus had grown for five days, only water exposed for 12 h or 24 h caused 100% immobility to *M. incognita* J_2. Both GLMS generated had all parameters significant by z test (< 2e-16). According to the estimates of GLMS coefficients, there was a positive correlation between percentage of immobile J_2 and increased time (√

(hours of exposure of J_2 to volatiles), in both periods of gas formation, 5 and 7 days. There was no immobility of J_2 in the control, where water was exposed to VOCs emitted by YES medium. In addition, no nematicidal activity was observed. The repetition of the assay is presented (experiment 1B) (Figure 1).

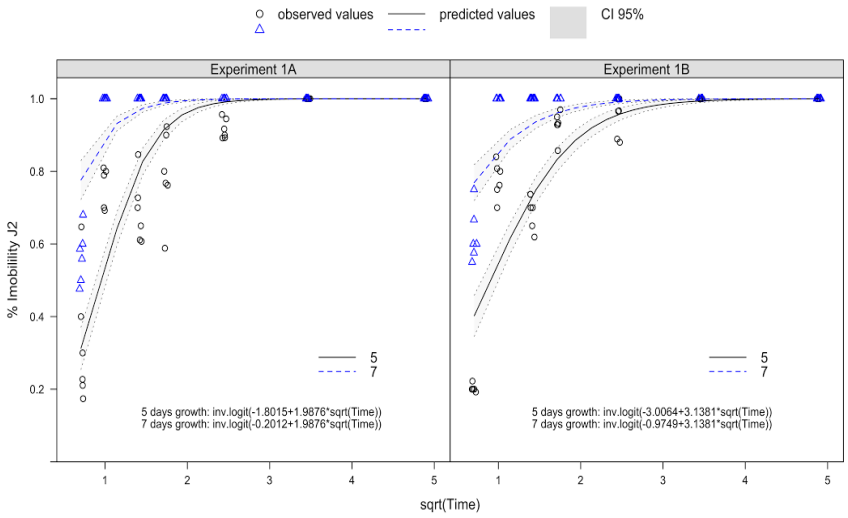

Figure 1. Toxicity of water exposed to volatile compounds of *Fusarium oxyspporum* 21 based on the percent immobility of *Meloidogyne incognita* second-stage juveniles (J_2). The water exposure time in the gas chamber (0.5 h, 1 h, 2 h, 3 h, 6 h, 12 h or 24 h) were transformed and expressed as the square root (sqrt). The assay was done twice (experiments 1A and 1B).

3.2 Infectivity and reproduction of *Meloidogyne incognita* in tomato after exposure of second stage juveniles to volatiles of *F.o-21* retained in water

The infectivity of *M. incognita* J_2 exposed to COVs retained in water decreased by 42 to 70% and by 37 to 65%, compared to the negative control (water) in two independent experiments 2.1 and 2.2, respectively (Figures 2A and 2C). Reproduction of *M. incognita* decreased by 37 to 65% and by 38 to 59%, compared to the same control

(Figures 2B and 2D). The J_2 exposed to carbofuran showed reduction in infectivity and reproduction by 85% and 77% and by 90% and 95% respectively, experiments 2.1 and 2.2. In the experiment 2.1, the number of eggs was statistically similar to carbofuran when J_2 were in contact with water exposed to gases for 1, 2, 6, and 12 h (Figure 2B).

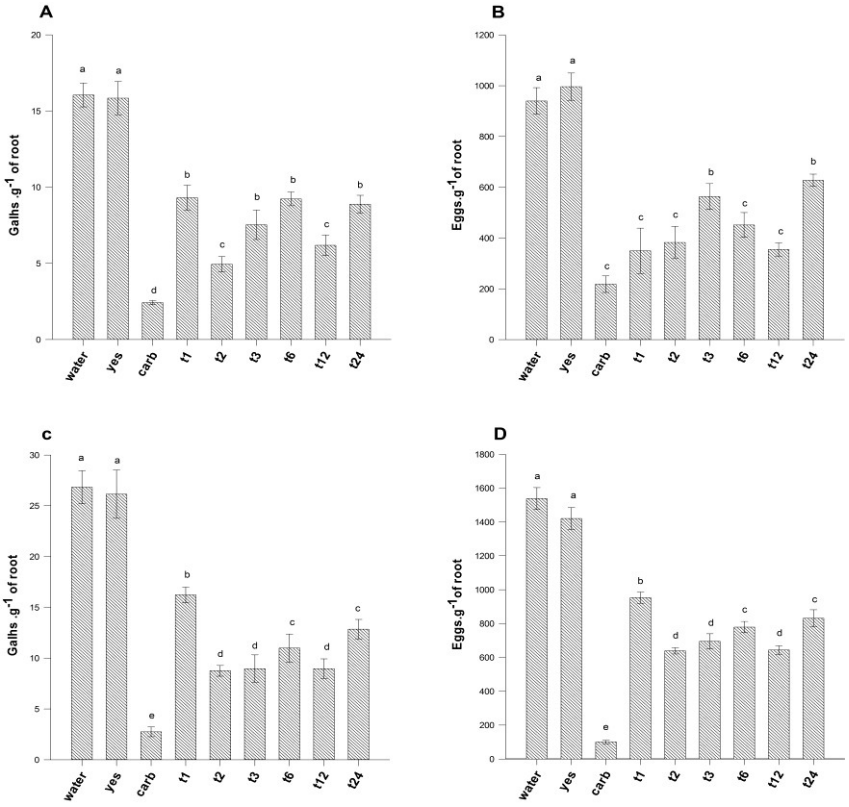

Figure 2. Number of galls and eggs per gram of tomato root 35 days after *Meloidogyne incognita* inoculation. Water was exposed for different periods of time 1 h (**t1**), 2 h (**t2**), 3 h (**t3**), 6 h (**t6**), 12 h (**t12**); 24 h (**t24**) to volatiles emitted by *Fo-21* - gas chamber 7 days. Then the second stage juveniles (J_2) were exposed for 24 h to this water. Vertical bars represent standard deviation. **2A** and **2B** assay 2.1; **2C** and **2D** assay 2.2.

3.3 Application of water exposed to volatiles directly to tomato plants inoculated with second stage juveniles of *Meloidogyne incognita*

When water exposed to VOCs of *F.o-21* was applied to the inoculation site of *M. incognita* J_2, infectivity and reproduction were significantly ($p < 0.001$) reduced compared to the negative control in the two independent experiments (Figure 4). Application of water exposed to VOCs reduced infectivity and reproduction of *M. incognita* by 34 and 32%, respectively, in the experiment 3A. In the experiment 3B, number of galls decreased by 56% and number of eggs by 49%. Weight of root systems did not differ among the treatments. The two independent experiments are presented (Figure 4).

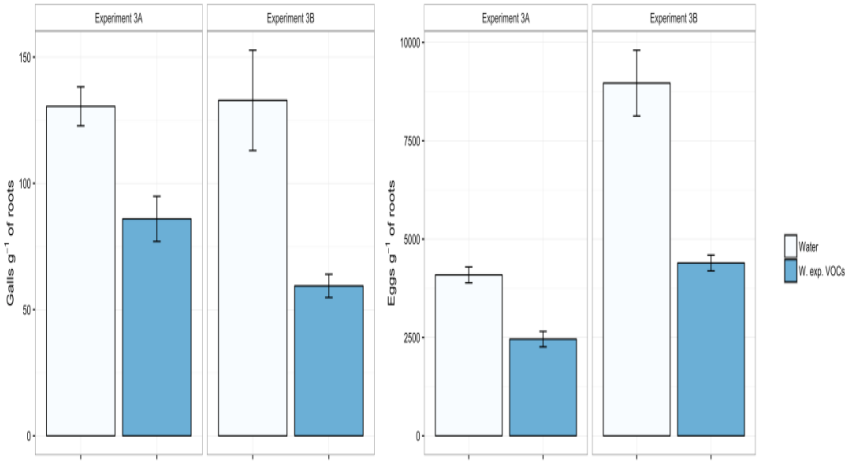

Figure 4. Number of galls and eggs per gram of tomato root 35 days after nematode inoculation. Two applications of water exposed to VOCs (W.exp. VOCs) were made in substrate infested with *M. incognita* second-stage juveniles (J_2), the first applications at the time of the infestation with J_2 and the other one after 24 h. Vertical bars represent standard deviation. Experiments 3A and 3B.

3.4 Identification of volatile compounds emitted by *Fusarium oxysporum* and retained in water by GC-MS

Analysis of VOCs by GC-MS indicated that the major classes of compounds emitted by *Fo-21* are sesquiterpenes, esters and alcohols. In water exposed to VOCs 28 compounds were identified; while in emissions of the fungus 36 compounds were identified (Table 1). Most molecules emitted by the fungus were dissolved in water exposed to VOCs. Although the molecules 1-propanol, ethyl propanoate, propyl acetate, 2-methylpropyl acetate, and 4-heptanone were present in water, they were not detected in the air of the gas chamber. Conversely, the molecules 2,3-butanediol, 2-methylbutanoic acid, furfuryl alcohol, 3-methylthio-propanol, benzyl alcohol, phenylethyl alcohol, octyl acetate, decanol, nonyl acetate, sesquiterpene, decyl acetate, dodecanol, ethyl dodecanoate, and tetradecanol were detected in the air of the gas chamber but not in the water exposed to VOCs. The peak areas of ten molecules were larger in water exposed to VOCs, which were ethanol, ethyl acetate, 3-methyl-1-butanol, 2-methylpropyl acetate, 3-methylbutyl acetate, 2-methylbutyl acetate, β-acorenol, two sesquiterpenes and one unidentified compound. In the air of the gas chamber nine compounds were detected in higher concentrations: ethanol, ethyl acetate, 3-methyl-1butanol, 3-methylbutyl acetate, phenylethyl alcohol, an unidentified sesquiterpene, α arcoradieno, dodecanol, and one unidentified molecule. We could not identify some sesquiterpenes but recognized their mass spectrum fragmentation as oxygenated sesquiterpens.

Three compounds were not identified in the mass spectral library. However, based on the mass spectrum we could say that they are closely related compounds, differing only by a methyl group and its

position in the molecule. The mass spectra of these three compounds are shown in the supplemental material. Analysis of VOCs by GC-MS indicated little variation in the profile of volatiles in water in different periods of exposure to the gas chamber formed by VOCs emitted by *Fo-21,* with little change in the peak area of each compound depending on exposure time.

Table 1. VOCs identified by SPME-GC-MS.

	Compound	RI	RI	water	In the
Alcohols					
1	Ethanol	x	x	vv	vv
2	1-propanol	x	x	v	
3	3-Methyl-1-butanol	729	734	vv	vv
4	2-Methyl-1-butanol	734	738	x	v
5	2,3-butanediol	800	811		v
6	Furfuryl alcohol	865	866		v
7	2-heptanol	903	905	v	v
8	3-Methyl-thiopropanol	977	978		v
9	Benzyl alcohol	1038	1032		v
10	Phenylethanol	1112	1116		vv
11	Decanol	1273	1272		v
12	Dodecanol	1470	1470		vv
13	Tetradecanol	1679	1671		v
14	p-ethylanisole	1115	1122	v	v
Aldehyde					
1	Acetaldehyde	x	x	v	v
Esters					
1	Phenylethyl acetate	1253	1257	v	v
2	Decyl acetate	1406	1408		x
3	Nonyl acetate	1309	1302		x
4	Ethyl acetate	609	608	vv	vv
5	Ethyl propanoate	708	709	v	
6	Propyl acetate	711	712	v	
7	2-methylpropyl acetate	769	776	vv	
8	3-Methylbutyl acetate	876	876	vv	vv
9	2-Methylbutyl acetate	879	885	vv	v
10	p-methylanisole	1020	1020	x	v
11	Octyl acetate	1208	1211		v
12	2-methylethyl butanoate	843	846	v	v
13	3-methylethyl butanoate	850	856	v	v
14	Ethyl phenylacetate	1241	1244	v	v
15	Ethyl dodecanoate	1590	1593		v
16	2-Methyl-2-butenyl acetate	938	949	v	v
17	2,3-butanediol acetate	1067	x	v	v
Acid					
1	2-Methylbutanoic acid	859	x		v
ketone					
1	4-Heptanone	870	869	v	
2	2-heptanone	889	889	v	v
3	2-nonanone	1093	1091	v	v
Sesquiterpene					
1	α cedrene	1414	1411	v	v
2	ß cedrene	1421	1420	v	v
3	α acoradiene	1461	1466	v	vv
4	Nerolidol	1556	1563	x	v
5	ß acorenol	1637	1637	vv	v
6	Sesquiterpene[b]	1498	x	v	v
7	Sesquiterpene[b]	1506	x	v	v
8	Sesquiterpene[b]	1355	x	v	v
9	Sesquiterpene[b]	1373	x		v

10	Sesquiterpene[b]	1436	x	vv	vv
11	Sesquiterpene[b]	1619	x	vv	v
Others					
1	Unidentified	1166	x	v	v
2	Unidentified	1174	x	v	v
3	Unidentified	1195	x	vv	vv

v -Compound present in the sample; vv—major compound.

[a] Theoretical retention indexes according to the literature (R.P. Adams, Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th Publishing Corp., Carol Stream, 2007.) (http://webbook.nist.gov/chemistry/)(Jens Rohloff, Atle M. Bones; Phytochemistry 66 (2005) 1941–1955).

[b] Compounds not identified due to their low purity or low peak intensity in the mass spectrum. Mass spectrum fragmentation typical of non-oxygenated sesquiterpenes.

4 DISCUSSION

This is the first experiment *in vivo* of water exposed to VOCs emitted by microorganisms that reduces infectivity and reproduction of a PPN. As plastic covering was not used at the application site, possibly the addition of a synthetic mixture similar to volatiles emitted by *Fo-21* in irrigation water could cause toxicity to PPN in the soil, also decreasing their potential infectivity and reproduction. Application of water exposed to VOCs of *Fo-21* in substrate infested with *M. incognita* J_2 confirmed the toxicity acquired by water and suggests that VOCs emitted by microorganisms traveling through soil can intoxicate PPN, as proposed by Wheatley, 2002. There has only one study showing toxicity of water exposed to VOCs emitted by microorganisms to nematodes (Grimme *et al*., 2007).

To the best of our knowledge, this is the first identification of the molecule profile retained in water exposed to volatiles emitted by a microorganism and toxic to a PPN. A diverse mixture of volatile molecules were identified both in the air and dissolved in water, confirming studies of emission of VOCs by other microorganisms (Gu *et*

al., 2007; Siddiquee *et al*., 2012; Naznin *et al*., 2013; Garbeva *et al*., 2014; Li *et al*., 2015). Retention of VOCs in water is related to the specific solubility of each molecule. As water is polar, it is expected that polar compounds emitted have greater solubility due to intermolecular interactions, such as hydrogen and dipole-dipole bonds. Polar molecules of VOCs are more strongly retained (dissolved) in water than aromatic and aliphatic compounds (Ruiz *et al*., 1998).

Many of the compounds emitted by *F.o-21* have shown nematicidal activity (Gu *et al*., 2007; Huang et al., 2010; Kang et al., 2013; Barros et al., 2014a). Some compounds emitted by *F.o-21* are referred to as inducers of growth, disease resistance and drought tolerance (Cortes-Barco *et al*., 2010; Ryu *et al*., 2004; Cho *et al*., 2008).

Apparently, the concentration of VOCs dissolved in water or exposure time of J_2 to VOCs were below the nematicidal threshold. The 24 h exposure of *M. incognita* J_2 to the gas chamber formed by volatiles from *M. albus* or macerated broccoli caused high immobility but not mortality (Grimme *et al*., 2007; Barros *et al.,* 2014b). However, 72 h exposure to VOCs from *F.o-21* resulted in high mortality of *M. incognita* J_2 (Freire *et al*., 2012). Mitchell *et al*., 2007, demonstrated that the cuticle of free-living nematode *Caenorhabditis elegans* is not a barrier for ethanol entry within the nematode body and that ethanol has an anesthetic effect on nematodes at the concentrations tested. As ethanol is a volatile emitted at higher concentrations by *Fo-21*, the lack of a barrier restricting ethanol entry within the nematode body may be responsible for rapid immobility of J_2 and subsequent recovery of mobility.

Decrease in infectivity and reproduction of *M. incognita* J_2 exposed to volatiles retained in water showed that, although VOCs caused no mortality, they affected the ability of J_2 to cause disease. Exposure to anesthetics of the first larval stage (L1) of *C. elegans* resulted in adults with nerve damage and altered chemotaxis (Gentry *et al*., 2013). This may be the reason why *M. incognita* J_2 exposed to VOCs of *F.o-21* have low efficiency in causing disease and low fertility. Damage to the nervous system of nematodes may interfere with their ability to find host and locate appropriate cells to form feeding site and reproduction partners (McCarter, 2008).

The perforation in the I-plate lid allowed short-term water exposure to gas chamber formed with diverse VOCs emitted during *F.o-21* growth. So far, the routine procedure has been opening the plate after fungal colony growth to add the test nematode, thus losing VOCs already emitted (Grimme *et al*., 2007; Freire *et al*., 2012; Costa *et al*., 2015). Formation of gas chamber with VOCs of *Fo-21* made water toxic to J_2 even in short-term exposure. This change opens up prospects for use in tests on short-term exposure (minutes) with J_2 of *Meloidogyne* spp. in this same array of volatiles. Long-term storage of J_2 in water caused relevant body energy losses, which resulted in infectivity and reproduction loss (Rocha *et al*., 2015).

From the identification of molecules retained in water, other studies can be developed to test their toxic nature or induction to resistance to plant diseases, as performed by Gu *et al*., 2007 and Naznin *et al*., 2014). In addition, mixtures to enhance nematicide efficiency still need to be tested. A synthetic formulation based on VOCs emitted by *M.*

albus showed effectiveness in controlling *M. incognita* on tomatoes (Grimme *et al*., 2007).

As inoculum variability, shelf life and introduction of exotic microorganism in soil pose challenges for using formulations with biological agents, a synthetic formulation containing VOCs emitted by *Fo-21* could overcome the challenges and generate an effective nematicide. This study provides scientific references for exploring and applying volatiles emitted by *F. oxysporum* isolate *21* in the control of *M. incognita.*

5 ACKNOWLEDGMENTS

The authors gratefully acknowledge the Brazilian financial support provided by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Coordenacão, de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

6 REFERENCES

Adams, R.P., 2007. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry. (fourth ed.) Allured Publishing Corporation, Carol Stream, Illinois USA

Alpha, C.J., Campos, M., Jacobs-Wagner, C., Strobel, S.A., 2015. Mycofumigation by the volatile organic compound-producing fungus *Muscodor albus* induces bacterial cell death through DNA damage. Appl Environ Microbiol. 81, 1147–1156.

Asensio, D., Peñuelas, J., Filella, I., Llusià, J., 2007. On-line screening of soil VOCs Exchange responses to moisture, temperature and root presence. Plant soil. 291, 249-261.

Arthur, C. L., Pawliszyn, J., 1990. Solid-phase microextraction with thermal desorp-tion using fused silica optical fibers. Anal. Chem. 62, 2145–2148.

Banerjee, D., Pandey, A., Jana, M., Strobel, G., 2014. *Muscodor albus* MOW12 an Endophyte of *Piper nigrum* L. (Piperaceae) Collected from North East India Produces Volatile Antimicrobials. Indian J Microbiol. 54, 27–32.

Cortes-Barco, A.M., Goodwin, P.H., Hsiang, T., 2010. Comparison of induced resistance activated by benzothiadiazole, (2R,3R)-butanediol and an isoparaffin mixture against anthracnose of *Nicotiana benthamiana*. Plant Pathology. 59, 643-653.

Barros, A.F., Campos, V.P., Silva, J.C.P., Pedroso, M.P., Medeiros, F.H.V., Pozza, E.A., Reale, A.L., 2014a. Nematicidal activity of volatile organic compounds emitted by *Brassica jucea, Azadirachta indica, Canavalia ensiformis, Mucuna pruriens* and *Cajanus cajan* against *Meloidogyne incognita.* Applied Soil Ecology. 80, 34-43.

Barros, A.F., Campos, V.P., Silva, J.C.P., López, L.E., Silva, A.P., Pozza, E.A., Pedroso, L.A., 2014b. Tempo de exposição de juvenis de segundo estádio a voláteis emitidos por macerados de nim e de mostarda e biofumigação contra *Meloidogyne incognita.* Nematropica. 44, 190-199.

Busko, M., Kulik, T., Ostrowska, A., Goral, T., Perkowski, J., 2014. Quantitative volatile compound profiles in fungal cultures of three different *Fusarium graminearum* chemotypes. FEMS Microbiol. Lett. 359, 85-93.

Campos, V.P., Pinho, R.S.C., Freire, E.S., 2010. Volatiles produced by interacting microorganisms potentially useful for the control of plant pathogens. Ciência e agrotecnologia. 3, 525-535.

Cho, S.M., Kang, B.R., Han, S.H., Anderson, A.J., Park, J., Lee, Y., Cho, B.H., Yang, K., Ryu, C., Kim, Y.C., 2008. 2R, 3R-Butanediol, a Bacterial Volatile Produced by *Pseudomonas chlororaphis* O6, Is Involved in Induction of Systemic Tolerance to Drought in *Arabidopsis thaliana*. Mol Plant-Microbe Interact. 21, 1067–1075.

Costa, L.S.A.S., Campos, V.P., Terra, W.C., Pfenning, L.H., 2015. Microbiota from *Meloidogyne exigua* egg masses and evidence for the effect of volatiles on infective juvenile survival. Nematology.17,715–724.

Effmert, U., Kalderás, J., Warnke, R., Piechulla, B., 2012. Volatile Mediated Interactions Between Bacteria and Fungi in the Soil. J Chem. Ecol. 38, 665–703.

Fernando, W.G.D., Ramarathnam, R., Krishnamoorthy, A.S., Savchuk, S.C., 2005. Identification and use of potential bacterial organic antifungal volatiles in biocontrol. Soil Biology & Biochemistry. 37, 955-964.

Freire, E.S., Campos, V.P., Oliveira, D.F., Pohlit, A.M., Norberto, N.P., Faria, M.R., 2012. Volatile substances on the antagonism between fungi, bacteria and *Meloidogyne incognita* and potentially fungi for nematode control. Journal of Nematology. 44, 321-328.

Garbeva, P., Hordijk, C., Gerards, S., de Boer W., 2014. Volatiles produced by the mycophagous soil bacterium Collimonas. FEMS Microbiol. Ecol. 87, 639–649.

Gentry, K.R., Steele, L.M., Sedensky, M.M., Morgan, P.G., 2013. Early Developmental Exposure to Volatile Anesthetics Causes Behavioral Defects in *Caenorhabditis elegans*. Anesth Analg. 116,185–189.

Grimme, E., Zidack, N.K., Sikora, R.A., Strobel, G.A., Jacobsen, B.J., 2007. Comparison of *Muscodor albus* Volatiles with a Biorational Mixture for Control of Seedling Diseases of Sugar Beet and Root-Knot Nematode on Tomato. Plant Disease 91, 220-225.

Gordon, T.R., Martyn, R.D., 1997. The Evolutionary Biology of *Fusarium oxysporum*. Annu. Rev. Phytopathol. 35, 111–28.

Gu, Y.i., Mo, M.H., Zhou, J., Zou, C.S., Zhang, K.Q., 2007. Evaluation and identification of potential organic nematicidal volatiles from soil bacteria. Soil Biology & Biochemistry. 39, 2567–2575.

Hoff, J.T., Mackay, D., Gillham, R., Shlu, W.Y., 1993. Partitioning of Organic Chemicals at the Air-Water Interface in Environmental Systems. Environ. Sci. Technol. 27, 2174-2180.

Huang, Y., Xu, C., Ma, L., Zhang, K., Duan, C., Mo, M.H., 2010. Characterisation of volatiles produced from *Bacillus megaterium*

YFM3.25 and their nematicidal activity against *Meloidogyne incognita*. Eur J. Plant Pathol. 126,417–422.

Hussey, R.S., Barker, K.R., 1973. A comparison of methods for collecting inocula of *Meloidogyne* spp., including a new technique. Plant Disease Report. 57, 1025-1028.

Insam, H., Seewald, M.S.A., 2010. Volatile organic compounds (VOCs) in soils. Biol Fertil Soils. 46, 199–213.

Kanchiswamy, C.N., Malnoy, M., Maffei, M.E., 2015. Chemical diversity of microbial volatiles and their potential for plant growth and productivity. Frontiers in Plant Science 6, 1-23.

Kang JS, Kim E, Lee SH, Park IK. 2013. Inhibition of acetylcholinesterases of the pinewood nematode, *Bursaphelenchus xylophilus*, by phytochemicals from plant essential oils. Pestic. Biochem. Physiol. 105, 50−56.

Leff, J.W., Fierer, N., 2008. Volatile organic compound (VOC) emissions from soil and litter samples. Soil Biology & Biochemistry. 40,1629–1636.

Lemfack, M.C., Nickel, J., Dunkel, M., Preissner, R., Piechulla, B., 2014. mVOC: a database of microbial volatiles. Nucleic Acids Research 42,744–748.

Li, M., Wen, F., Ying, H., He, M.M., Qin, X.J., Qing, D.Y., Huang, F.D., Hui, Y.S., 2012. A strategy to discover potential nematicidal fumigants based on toxic volatiles from nematicidal bacteria**.** African Journal of Microbiology Research. 6, 6106-6113.

Li, X.Y., Mao, Z.C., Wu, Y.X., Ho, H.H., He, Y.Q., 2015 Comprehensive volatile organic compounds profiling of Bacillus species with biocontrol properties by head space solid phasemicroextraction with gas chromatography-mass spectrometry. Biocontrol Science and Technology 25, 132–143.

McCarter JP. 2008. Molecular Approaches Toward Resistance to Plant-Parasitic Nematodes**.** In: Berg, R. H and Taylor, C. G (eds) Cell Biology of Plant Nematode Parasitic - Plant Cell Monographs. 239-267p.

Mitchell, P.H., Bull, K., Glautier, S., Hopper, N.A., Holden-Dye, L., O'Connor, V., 2007. The concentration-dependent effects of ethanol on Caenorhabditis elegans behaviour. The Pharmacogenomics Journal. 7,411–417.

Naznin, H.A., Kimura, M., Miyazawa, M., Hyakumachi, M., 2013. Analysis of volatile organic compounds emitted by plant growth-promoting fungus *Phoma* sp GS8-3 for growth promotion effects on tobacco. Microbes Environ 28, 42–49.

Naznin, H.A., Kiyohara, D., Kimura, M., Miyazawa, M., Shimizu, M., Hyakumachi, M., 2014. Systemic resistance induced by volatile organic compounds emitted by plant growth-promoting fungi in *Arabidopsis thaliana*. PLoS One. 1, e86882.

NIST, 2013. NIST Chemistry Webook-National Institute of Standards and Technology. http://webbook.nist.gov/chemistry/. Accessed 30 June 2013.

Noling, J.W., 2002. The Practical Realities of Alternatives to Methyl Bromide: Concluding Remarks**.** Phytopathology. 92, 1373-5.

Oka, Y., 2010. Mechanisms of nematode suppression by organic soil amendments – A review. Applied Soil Ecology. 44, 101-115.

Riga, E., Lacey, L.A., Guerra, N., 2008. *Muscodor albus*, a potential biocontrol agent against plant-parasitic nematodes of economically important vegetable crops in Washington State, USA. Biological Control. 45, 380–385.

Rocha, F.S., Campos, V.P., Catão, H.C.R.M., Muniz, M.F.S., Civil, N., 2015. Correlations among methods to estimate lipid reserves of second-stage juveniles and its relationships with infectivity and reproduction of *Meloidogyne incognita*. Nematology, 17, 345-352.

Rohloff, J., Bones, A.M., 2005. Volatile profiling of Arabidopsis thaliana putative olfactory compounds in plant communication. Phytochemistry. 66, 1941–1955.

Ryu, C.M., Farag, M.A., Hu, C.H., Reddy, M.S., Kloepper, J.W., Paré, P.W., 2004. Bacterial volatiles induce systemic resistance in *Arabidopsis*. Plant Physiology. 134, 1017–1026.

Ruiz, J., Bilbao, R., Murillo, M.B., 1998. Adsorption of different voc onto soil minerals from gas phase: influence of mineral, type of voc, and air humidity. Environmental Science Technology. 32, 1079-1084.

Siddiquee, S., Cheond, B.E., Taslima, K., Kausar, H., Hasan, M.M., 2012. Separation and identification of volatile compounds from liquid cultures of *Trichoderma harzianum* by GC-MS using three different capillary columns. J Chromatogr Sci. 50, 358–367.

Sikora, R.A., Bridge, J., Starr, J.L., 2005. Management practices: An overview of integrated nematode management technologies. *Plant Parasitic Nematodes in Subtropical and Tropical Agriculture: Second Edition*. 793-825.

Strobel, G.A., Dirkse, E., Sears, J., Markworth, C**.,** 2001. Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. Microbiology. 147, 2943–2950.

Trifonova, Z., Karadjova, J., Georgieva T., 2009. Fungal parasites of the root-knot nematodes *Meloidogyne* spp. in southern Bulgaria. Estonian Journal of Ecology, 58, 47-52.

Trudgill, D.L., Block, V.C., 2001. Apomitic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. Annu Rev Phytopathol. 39, 53-77.

Wheatley, R. E., 2002. The consequences of volatile organic compound mediated bacterial and fungal interactions. Antonie van Leeuwenhoek, 81, 357–36.

http://bioinformatics.charite.de/mvoc/. Assessed 29 May 2016.

ARTIGO 2

Volatile organic molecules from *Fusarium oxysporum* exhibiting nematicidal activity to *Meloidogyne incognita* and *Heterodera schachtii*

Willian César Terra, Vicente Paulo Campos, Lilian Simara Abreu Soares Costa, Júlio Carlos Pereira da Silva, Aline Ferreira Barros, Geert Smant, Denilson Ferreira Oliveira

Preparado de acordo com as normas do periódico Crop Protection

Abstract

Volatile organic compounds (VOCs) produced by microorganisms are potential alternatives for the development of new nematicides. In a previous study we identified VOCs produced by *Fusarium oxysporum* isolate 21 (*F.o–21*). In the present study, the eight most abundant VOCs produced by *F.o-21* were tested against *Meloidogyne incognita* and *Heterodera schachtii*. Compounds 2–methylbutyl acetate **(1)**, 3-methylbutyl acetate **(2)**, ethyl acetate **(7)** and, 2-methylpropyl acetate **(8)**, caused *in vitro* mortality of 100%, 92.5%, 100% and 83.8%, respectively, in second-stage juveniles (J_2) of *M. incognita* and 90%, 88.8%, 96.3% and 97.5% mortality, respectively, on *H. schachtii* J_2, when tested at a concentration of 500 $\mu g.ml^{-1}$. The lethal concentration (LC_{50}) for compounds 1, **2**, **7** and **8**, in *M. incognita* J_2 was of 236, 198, 213 and 218 $\mu g.ml^{-1}$, respectively. Under the same conditions, the commercial nematicide carbofuran (2,3-dihydro-2,2-dimethyl-1-benzofuran-7-yl N-methylcarbamate) showed a LC_{50} of 191 $\mu g.ml^{-1}$. Eggs exposed to compounds **1**, **2**, **7** and **8**, for 72 h showed a reduction

in hatching of 36.5%, 55.2%, 91.0% and 10.3%, respectively, and reduction in infectivity of 53%, 53%, 32% and 39%, respectively. When compounds were applied in tomato seedlings infested by *M. incognita,* compound **1** reduced the number of galls per gram of root in 27.5% when compared to the negative control (without the application of nematicide).

Key words: bioprospection, volatiles, plant-parasitic nematodes, *Fusarium oxysporum*

1. INTRODUCTION

Plant-parasitic nematodes (PPN) are responsible for the reduction of approximately 10% of the world food production, representing a significant limiting factor for global agriculture (McCarter, 2008). Among these parasites are the root-knot nematodes, *Meloidogyne* spp., and cyst nematodes, *Heterodera* spp., which due to their wide distribution, allied to the vast host range and to their sophisticated parasitism process, are amongst the main PPN groups (Goverse & Smant, 2014).

Still nowadays commercial nematicides based on chemical products are one of the foremost tools used to control PPN. However, due to their extremely environmental harmful potential, these products have been removed from commercial trading, thus reducing practical control (Noling, 2002). Just to recall the difficulty in finding new molecules with nematicidal activity and potential for commercial usage, with the exception from abamectin, no other class of efficient nematicide was released commercially since the decade of 1970 (McCarter, 2008). In the U.S.A., a new nematicide, Nimitz®, originated form a new chemical class (Fluoro-alkenyle), received a register from

the United States Environmental Protection Agency (EPA) in March, 2015.

Among the potential molecules for the development of commercial nematicides, one must consider the volatile organic compounds (VOCs), which are naturally present in the ecology of plants and microorganisms (Campos *et al*., 2010). VOCs are characterized by having up to 20 carbon atoms, high vapor pressure and lipophilic character (Effmert *et al*., 2012). In the last years a considerable progress has been made in understanding the role of VOCs produced by microorganisms (mVOCs), in the multi-trophic interactions from the soil. As an example, non-pathogenic isolates of *Fusarium oxysporum* Schlecht release VOCs with antifungal, nematicidal and plant growth promoting effects (Freire *et al*., 2012; Zhang *et al*., 2015; Bitas *et al*., 2015; Costa *et al*., 2015). Another example is given by Gu *et al*. (2007), whom demonstrated nematicidal activity from nine VOCs from microbial origin on *Bursaphelenchus xylophilus* Steiner & Buhrer at a concentration lower than 2 $mmol.L^{-1}$. These and other previous studies available in the literature, with different objectives and microorganism, document near 1000 VOCs produced by 480 bacterial and fungal species and are described and gathered in a specific data base http://bioinformatics.charite.de/movc/ (Lemfack *et al*., 2014).

In general, studies regarding the activity of VOCs against nematodes use the second-stage juvenile (J_2), which is the infective stage of the nematode. Consequently, little is known about the effect of such compounds on eggs, which are the main surviving form for nematodes in the soil, between harvest seasons. As eggs exhibit an internal glycolipid layer resistant to external substances, protecting life

stages on the inside (Eisenback & Hunt, 2009), it is probable that a substance directly active against J_2 may not affect the nematodes inside eggs.

In face of the potential that VOCs exhibit to control PPN, initial studies were performed allowing to verify the production of VOCs with significant nematicidal activity by *F. oxysporum* isolate 21 (*F.o-21*) (Freire *et al*., 2012 & Terra *et al*., 2016 in press). Through the analysis by gas chromatography–mass spectrometry (GC-MS), 50 VOCs produced by this fungus were detected. In the present study we investigated the nematicidal activity of the eight most abundant VOCs produced by *F.o-21*. The objectives of the present study were: (1) to evaluate *in vitro* nematicidal activity from VOCs, in a single concentration, over *Meloidogyne incognita* J_2 (Kofoid & White) Chitwood and *Heterodera schachtii* Schmidt; (2) to calculate lethal concentrations for 50% (LC_{50}) of *M. incognita* J_2; (3) to evaluate the effect of VOCs in the eclosion of *M. incognita* J_2; (4) to test the infectivity of *M. incognita* J_2 originated from eggs exposed to VOCs in tomato plants; and (5) to test the infectivity of *M. incognita* J_2 in tomato plants after direct application of the compounds on infested substrate.

2 MATERIAL AND METHODS

2.1 Acquisition of VOCs

The eight VOCs used in the experiments were 2-methylbutyl acetate **(1)**, 3-methylbutyl acetate **(2)**, 1-phenylethanol **(3)**, (R,R)-butane-2,3-diol **(4)**, Ethanol **(5)**, 3-methyl-1-butanol **(6)**, Ethyl acetate **(7)**, 2-methylpropyl acetate **(8)**. They were obtained from commercial sources and used according arrival, without any previous treatment. These corresponded to the most abundant VOCs produced by *F.o-21*

(Terra *et al*., 2016). The commercial availability and costs were also considered in order to choose the VOCs.

2.2 Collecting *M. incognita* eggs and J_2

Pure populations of *M. incognita* were multiplied in tomato plants and kept in greenhouse for three months. Then, galled roots were separated from soil, washed in tap water and cut in pieces of 0.5 cm length. Form these fragments, eggs were extracted according to the process described by Hussey & Barker (1973). Eggs were used in the experiments or set in a hatching chamber. All J_2 hatched between the second and third days were used in the experiments. Cysts of *H. schachtii* were obtained from infested cabbage roots. These cysts were set in a hatching chamber with a solution of ZnSO4 at a concentration of 3.14 $mmol.L^{-1}$. After 72 h, hatched J_2 were collected to perform the toxicity assay.

2.3 Culture of *F. oxysporum*

Isolate *F.o-21*, obtained from *Meloidogyne exigua* Goeldi egg's mass was selected for the experiments performed in this work due to exhibiting the capacity to produce toxic VOCs against *M. incognita* (Freire *et al*., 2012). The isolate is preserved under the under number (CML 3605) in the Coleção Micologica de Lavras at the Federal University of Lavras, MG, Brazil, preserved on water (Castellani's method). In the experiments, the isolate from the stock culture was transferred to malt agar medium (MA) (malt 20 $g.L^{-1}$, agar 20 $g.L^{-1}$) and incubated for 6 days at 25^0 C. Then, a 10 mm plug was taken from the border of the colonies and sub cultured to new plates with MA medium, where the fungus was kept at 25^0 C until used in the essays.

2.4 Nematicidal activity of VOCs on *M. incognita* and *H. schachtii*

In order to evaluate the nematicidal activity of the VOCs against *M. incognita* and *H. schachtii* J_2, an aqueous suspension (200 µl) containing 250 J_2 and a solution (1 ml) of the VOC to be tested, at a concentration of 600 $\mu g.ml^{-1}$ in aqueous solution with Tween 80® at $0{,}01 g.ml^{-1}$, were set in a polypropylene micro-tube (2 ml). In this way J_2 were exposed in direct contact to a solution of the VOCs at 500 $\mu g.ml^{-1}$ concentration. Micro-tubes were closed with screw cap and sealed with plastic film Parafilm®. A completely random design with five replicates/treatment was used. The positive control consisted in the exposure of J_2 to an aqueous solution of carbofuran (2,3-dihydro-2,2-dimethyl-1-benzofuran-7-yl N-methylcarbamate, Aldrich, 98%) at 500 $\mu g.ml^{-1}$. The negative control was the solution used for dissolution of the compounds, Tween 80® at $0.01 gm.L^{-1}$.

After 1h or 24 h (independent essays) of incubation at 28^0C, an evaluation of the mobility and mortality of J_2 was performed as described by Chen & Dickson (2000) and modified by Amaral *et al*. (2003). Only VOCs that resulted in mortality of *M. incognita* J_2 above 80% were selected for the lethal concentration (CL_{50}) and following assays.

With the objective to determine the corresponding lethal concentrations for 50% (LC_{50}) of the *M. incognita* J_2, the selected VOCs were again evaluated at nine different concentrations. The compounds tested were: 2-methylbutyl acetate **(1)**, 3-methylbutyl acetate **(2)**, ethyl acetate **(7)** and 2-methylpropyl acetate **(8)**. The nematode's J_2 were exposed to concentrations of 500, 400, 350, 300, 275, 225, 200, 150 and 100 $\mu g.ml^{-1}$. A completely random design with five replicates/treatment

was used. The exposure period for J_2 to the VOCs was of 48 h. The values for LC_{50} to carbofuran were also determined.

2.5 Hatching of *M. incognita* J_2 from eggs exposed to VOCs

In a hatching chamber, constituted by a Petri dish with 6 cm diameter, 2 ml of an aqueous suspension containing 5000 *M. incognita* eggs and VOC's solution (10 ml) with 600 $\mu g.ml^{-1}$ in aqueous solution of Tween 80® at 0.01g.ml^{-1}, were placed. In that way, the final concentration of VOCs within the chamber was of 500 $\mu g.ml^{-1}$. The tested compounds here are 2-methylbutyl acetate **(1)**, 3-methylbutyl acetate **(2)**, ethyl acetate **(7)** and 2-methylpropyl acetate **(8)**. The chamber was closed with a cover and sealed with plastic film (Parafilm®). Then, hatching chambers containing eggs were incubated at 28 ^{0}C in the darkness. A completely random design with five replicates/treatment was used. The positive control consisted of carbofuran at 600 $\mu g.ml^{-1}$ (final concentration in the chamber: 500 $\mu g.ml^{-1}$). The negative control was the solution used for dissolution of the compounds, Tween 80® at 0.01g.ml^{-1}. After 72h, the hatching chamber was opened to identify hatched J_2. Residual eggs in the hatching chamber were washed in tap water and set in the hatching chambers filled with tap water. After 72h, the emerged J_2 were quantified again.

An additional treatment consisted in the exposure of *M. incognita* eggs to the VOCs produced by *F.o-21*. This was accomplished using a two-compartmented Petri dishes (100 mm x 15 mm) made of plastic that contained a center partition (I-plates) described by Fernando *et al*., (2005). In the surface of a plate cover (opposite side to the fungus culture), a perforation of 2 mm diameter was made, enough to allow the

insertion of a hypodermic needle (5 ml), which was sealed using adhesive tape Adelbras®. The hole was used to add eggs into the plates without removing the covers and consequently losing the VOCs produced during the first days of fungal growth. The substrate used for fungal growth was yeast extract sucrose agar (YES), (yeast extract 20 $g.L^{-1}$, sucrose 150 $g.L^{-1}$, $MgSO_4.7H_2O$ 0.5 $g.L^{-1}$, agar 20 $g.L^{-1}$), as recommended for the analysis of secondary metabolites by the Centraal Bureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre (Busko *et al*., 2014). One half of the I-plates was filled with YES medium where a plug of 10 mm of the *F.o-21* was set. Then, plates were closed and sealed with plastic film Parafilm®. Plates were incubated at 28^0C in the darkness. After a 5 days incubation period, 4 ml of a suspension containing 5000 eggs were injected in the compartment adjacent to the fungal culture. After removal of the hypodermic needle used for injection, the hole was sealed using adhesive tape. In that way, the suspension containing eggs was introduced inside a chamber containing the volatiles produced by *F.o*-21 during the first five days of incubation. Eggs introduced into such chamber were exposed during 72 h to the volatile compounds produced by *F.o-21*. After that, the chamber was opened to quantify the number of hatched *M. incognita* J_2.

2.6 Infectivity of *M. incognita* in tomato after exposure of eggs to VOCs

An aqueous suspension (2 ml) containing approximately 9.000 *M. incognita* eggs were set in glass flasks (30 ml). Then, flasks were added with 10 ml of a solution at 600 $\mu g.ml^{-1}$ in Tween 80® (0,01$g.ml^{-1}$), (final concentration in the flasks: 500 $\mu g.ml^{-1}$) of the compounds to be tested 2-methylbutyl acetate **(1)**, 3-methylbutyl acetate **(2)**, ethyl

acetate **(7)** and 2-methylpropyl acetate **(8)**. Flasks were closed with cover and sealed with plastic film (Parafilm®). Five replicates/treatment were used and as a positive control eggs were exposed to an aqueous solution of carbofuran (Aldrich, 98%), at a final concentration of 500 $\mu g.ml^{-1}$. The negative control consisted of the solution used for dissolution of the compounds (Tween 80® at 0,01 $g.ml^{-1}$). The flasks were incubated at 28^0C in the darkness. After 72 h the flasks were shake to homogenize the eggs in the solution and then opened. With the aid of a pipette, part (4 ml) of each suspension, containing approximately 3.000 eggs was equally distributed in four holes around the steam of tomato (*Solanum lycopersicum* L. cv. Santa Clara) seedlings. Each inoculated plant was 30 days old after sowing. Plants were growth in Styrofoam trays with 72 cells filled with Plantmax® substrate. Leaf fertilization was performed on inoculated plants at 7 day intervals by using leaf fertilizer Biofert®. After 35 days in greenhouse conditions the aerial portion was cut and discarded. Then, roots where separated from the substrate in still water and carefully washed to avoid losing fine roots. Roots were then set over towel paper for 20 minutes in order to eliminate water excess, before roots being weighted and the galls per radicular system determined.

2.7 Control of *M. incognita* in tomato by VOCs

The compounds tested and their concentrations were as follows: 2-methylbutyl acetate (**1**, 448 $\mu g.ml^{-1}$), 3-methylbutyl acetate (**2**, 376 $\mu g.ml^{-1}$), ethyl acetate (**7**, 404 $\mu g.ml^{-1}$) and 2-methylpropyl acetate (**8**, 414 $\mu g.ml^{-1}$). The aqueous solution of Tween 80® at 0.01 $\mu g.ml^{-1}$ was used as a negative control. As a positive control carbofuran was used at a concentration of 363 $\mu g.ml^{-1}$. All compounds were dissolved in Tween

80® at 0.01 µg.ml^{-1} until the concentration tested, corresponding to 1.9 times the valued of the LC_{50} for each of them.

Tomato seedlings growth in plastic pots (2 L) containing substrate Plantmax®, with 30 days old after sowing, were inoculated with an aqueous suspension of 4 ml containing 800 *M. incognita* J_2. The suspension containing the juveniles was equally distributed in four holes around the steam of the tomato seedlings and then the treatment solutions (4 ml) were also distributed in the holes. Plants were kept for 30 days in climatic chamber at 28^{0}C and 12 h photoperiod. At the end of this essay the aerial portion of the plants was cut and discarded. Roots were separated from the substrate in still water and carefully washed to avoid losing fine roots. Then roots were set over towel paper for 20 minutes in order to eliminate water excess, before roots being weighted and the galls per radicular system determined.

2.8 Statistical analysis

All assays were done twice observing the consistence of the results and set in a completely random design with five replicates per treatment. The results were previously submitted to normality test (Shapiro- Wilk) and error variance homogeneity (Bartlett). Once these assumptions were fulfilled the F test was applied through a variance analysis (ANOVA). When the F test was significant (P <0.05), mean values from treatments were compared by the Scott-Knott test (P <0.05). To calculate the LC_{50} values of mortality were transformed in percentage of death J_2 and submitted to a nonlinear regression.

3 RESULTS

3.1 Nematicidal activity of VOCs on *M. incognita* and *H. schachtii*

Amongst the eight compounds tested at a concentration of 500 µg.ml^{-1}, only **1**, **2**, **7** and **8** caused mortality of *M. incognita* J_2 equal or above 83.8% after 24 h exposure. Nematicidal activities of the other compounds were inferior to 9.0%. When the exposure time of *M. incognita* J_2 to the compounds was reduced to 1 h, compounds **2** and **8** still caused mortality above 71% (Table 1). Concerning to the *H. schachtii* J_2, similar results were obtained. The most active substances were also **1**, **2**, **7** and **8**, with mortality above 88% after 24 h of exposure, but substance **6** had nematicidal activity of 53,8 which was not observed with *M. incognita*.

Values of LC_{50} for **1**, **2**, **7** and **8**, against *M. incognita* J_2, were respectively of 236, 198, 213 and 218 µg.ml^{-1}. Within the same condition carbofuran showed a LC_{50} of 191 µg.ml^{-1}.

Table 1. Mortality of *Meloidogyne incognita* and *Heterodera schachtii* second stage juveniles (J_2), after exposure for 1 or 24 hours to a solution of volatile organic compounds. Exposure concentration was of 500 µg.ml^{-1}.

	J2 killed (% ± σ [a])		
	1 h	24 h	24 h
Compounds	*M. incognita*		*H. schachtii*
(1) 2-methylbutyl acetate	17,3 ± 6,5	100 ± 0	90,0 ± 3,5
(2) 3-methylbutyl acetate	84,0 ± 6,2	92,5 ± 2,5	88,8 ±3,1
(3) 1-phenylethanol	0 ± 0	3,8 ± 3,7	1,7 ± 2,0
(4) (R,R)-butane-2,3-diol	0 ± 0	2,5 ± 1,2	0,0 ± 1,0
(5) Ethanol	0 ± 0	1,3 ± 2,0	1,7 ± 2,0
(6) 3-methyl-1-butanol	0 ± 0	8,8 ± 6,4	53,8 ± 8,2
(7) Ethyl acetate	0 ± 0	100 ± 0	96,3 ± 1,3
(8) 2-methylpropyl acetate	71,3 ± 5,3	83,8 ± 2,0	97,5 ± 2,0
Tween 80	0 ± 0	0,4 ± 1,0	0,0 ± 0,0
Carbofuran	----	96,7 ± 3,0	----

[a] Standard deviation

3.2 Hatching of *M. incognita* J_2 from eggs exposed to VOCs

Exposure of *M. incognita* eggs to a solution of VOCs for 72 h resulted in a significant reduction ($p<0,001$) in the number of hatched J_2 for all treatments when compared to the negative control (Tween 80®) (Figure 1A). Compound **7** was the most active, reducing 91% of J_2 eclosion. Exposure to the VOCs produced by ***F.o-21*** resulted in a reduction of 82% of J_2 eclosion. The positive control, carbofuran, reduced eclosion in 15% when compared to Tween 80®. It is important to emphasize that all J_2 ecloded from eggs exposed to compounds **1**, **2**, **7**, **8** and carbofuran showed immobility.

When unhatched eggs exposed to volatiles compounds were poured in pure water, the highest number of hatched J_2 was observed in eggs exposed to compound **7** with a value that was 137% higher compared to the number of J_2 hatched in the negative control. The number of hatched J_2 in pure water in the positive control was statistically equal when compared to the negative control. The lowest numbers of hatched J_2 were observed in treatments with the compound **1** and ***F.o-21*** (Figure 1B). It must be emphasized that J_2 obtained in residual eggs from carbofuran (positive control) showed reduced mobility when compared with other treatments. By the sum of all hatched J_2, from direct contact to the compounds and in pure water it is possible to observe that treatments **(1)**, **(2)** and ***Fo-21*** reduced eclosion in 28.5%, 17.9% and 61.5% respectively, when compared to the negative control (Tween 80). Compound **8** increased eclosion in 9%, while compound **7** and carbofuran had similar results to Tween 80 (Figure 1C).

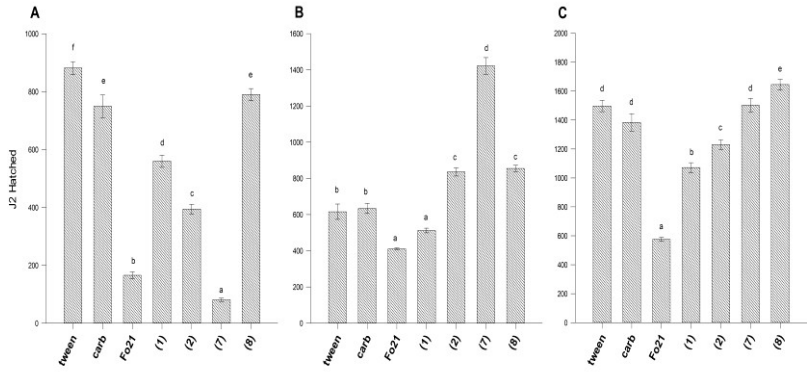

Figure 1. Hatching of *Meloidogyne incognita* second stage juveniles (J_2) after exposure to: solutions of 2–methylbutyl acetate **(1)**, 3-methylbutyl acetate **(2)**, ethyl acetate **(7)** and, 2-methylpropyl acetate **(8)**, at a concentration of 500 $\mu g.ml^{-1}$, volatiles from *Fusarium oxysporum* isolate 21 (***Fo21***); negative control ***Tween*** (Tween 80 ® at 0,01 $g.ml^{-1}$); and positive control ***carb*** (carbofuran at 500 $\mu g.ml^{-1}$). **A.** J_2 eclosion after exposure for 72 h. **B.** J_2 hatching from eggs remaining from **A** that were washed in water and set again in hatching chamber without treatments. **C.** Sum of **A** and **B**. Vertical bars represent standard error.

3.3 Infectivity of *M. incognita* in tomato after exposure of eggs to VOCs

Significant reduction ($p<0,001$) in the number of galls per tomato's root system was obtained when eggs were exposed to all tested compounds **1**, **2**, **7** and **8** compared to negative control (Tween). However, compounds **1** and **2** had the highest reduction in the number of galls about 53% when compared to the negative control (Tween 80), but lower than carbofuran (Figure 2).

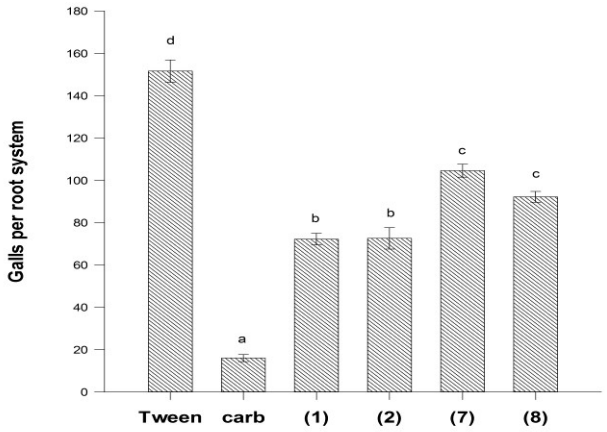

Figure 2. Number of galls per tomato's root system 30 days after *Meloidogyne incognita* eggs inoculation. *M. incognita* eggs were exposed to a solution of the compounds: 2–methylbutyl acetate **(1)**, 3-methylbutyl acetate **(2)**, ethyl acetate **(7)** and, 2-methylpropyl acetate **(8)**, during 72 h, at a concentration of 500 $\mu g.ml^{-1}$. After exposure, eggs were inoculated in tomato seedlings. Carbofuran at a concentration of 500 $\mu g.ml^{-1}$ was used as a positive control (**carb**) and Tween 80 at 0,01 $g.ml^{-1}$ was used as negative control (**tween**). Vertical bars represent standard error.

3.4 Control of *M. incognita* in tomato by VOCs

The lowest number of galls per gram of tomato root was observed when J_2 were exposed to carbofuran (positive control) (Figure 3). Treatments with compounds **2**, **7** and **8** were not statistically different

from the negative control (Tween 80). Regarding to the compound **1**, it significantly reduced the number of galls per root weight when compared to the negative control, resulting in intermediary values between the positive and negative control.

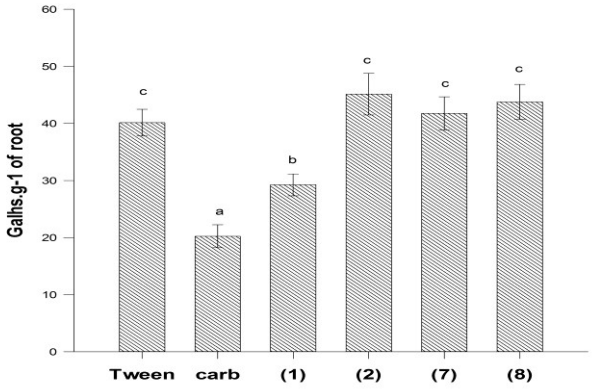

Figure 3. Number of galls per gram of tomato roots 30 days after *Meloidogyne incognita* J_2 inoculation. Compounds 2–methylbutyl acetate **(1)**, 3-methylbutyl acetate **(2)**, ethyl acetate **(7)** and, 2-methylpropyl acetate **(8)** were applied at concentrations of 448, 378, 404, 414 $\mu g.ml^{-1}$, respectively, at the moment of tomato's inoculation with *M. incognita* J_2. Carbofuran at a concentration of 363 $\mu g.ml^{-1}$ was used as a positive control (**carb**). Tween 80 at 0,01 $g.ml^{-1}$ was used as negative control (**tween**). Vertical bars represent standard error.

4 DISCUSSION

Previous studies have demonstrated that *F. oxysporum* non-pathogenic isolates produce VOCs that, as a whole, show nematicidal activity (Freire *et al*., 2012; Costa *et al*., 2015). However, once the profile of VOCs has been identified it is essential to evaluate the individual activity of each compound over its biological target. Consequently, this study demonstrated for the first time that four VOCs produced by *Fo-21*, when isolated and at a 500 $\mu g.ml^{-1}$ concentration, exhibit nematicidal activity above 80% against extremely important PPN. Such results verify the previous statement by Li *et al*. (2012), according whom VOCs produced by microorganisms are potential sources for the development of commercial efficient nematicides.

Esters (**1**, **2**, **7** and **8**) are amongst the main VOCs produced by *F. oxysporum* (Terra *et al* in press), and were the most active against nematodes in the present study, showing nematicidal activity above 80% on *in vitro* tests. This result is in agreement with the results from Seo *et al*. (2014), whom reported five esters exhibiting nematicidal activity against *B. xylophilus* of 98% at a concentration of 1000 $\mu g.ml^{-1}$. In addition, compounds **1** and **7** are usually found in macerates from plants being toxic against *M. incognita* J_2 (Barros *et al*., 2014a,b). Values of LC_{50} for compounds **1, 2,** **7** and **8** observed in the present work were close to the value of the positive control (carbofuran) which was of 191 $\mu g.ml^{-1}$. Values for LC_{50} above 400 $\mu g.ml^{-1}$ for esters in *M. incognita* were observed by Aissani *et al*., 2015. Seo *et al*., 2014 found five esters with values of LC_{50} under 48 $\mu g.ml^{-1}$ when tested in *B. xylophilus*

Alcohols (**3**, **4**, **5** and **6**) are also among the main VOCs produced by *F. oxysporum* (Terra *et al* in press). However, nematicidal

activity of alcohols in the present study was under 9%, excepting compound **6** which showed nematicidal activity above 53% for *H. schachtii* J_2. This is the most abundant volatile compound produced by *Muscodor albus*, VOCs compounds produced by this fungus are capable to kill tens of plant pathogenic fungi (Strobel *et al*., 2001). Compound **(4)**, studied in this work, despite not having showed any nematicidal activity is reported as an inductor of resistance in plants against plant pathogens (Cho *et al*., 2008 & Cortes-Barco *et al*., 2010).

In general, researchers have prioritized the use of J_2 in bioprospecting tests (Gu *et al*., 2007; Grimme *et al*., 2007; Freire *et al*., 2012, Barros *et al*., 2014a; Seo *et al*., Aissani *et al*., 2015; Costa *et al*., 2015), which can be at certain degree, understood as an error, once eggs correspond to the majority of the nematode's population in the field in the absence of the host. Consequently, the present study evaluated also the effect of VOCs on eggs. Exposure of *M. incognita* eggs to compounds **1**, **2** and especially compound **7** inhibited the eclosion of J_2, the inhibition also occurred when eggs were exposed to volatiles produced by *F.o-21* (Figure 1A). However, these compounds appear at a concentration of 500 $\mu g.ml^{-1}$ to be unable to kill the embryos inside the eggs, once a significant increase on J_2 eclosion was observed after eliminating the contact between eggs and the compounds (Figura 1B). Results also suggested that volatile compounds produced by *F.o-21* kill the embryos or delay eclosion of J_2. The evidences suggest that the combination of VOCs produced by *Fo-21* is more efficient than the individual effect of the constituent compounds of the mixture, confirming the reports from Strobel *et al*., 2001. The positive control (carbofuran) did not affect J_2 eclosion, in agreement with results

observed by Payan *et al*., 1987, whom demonstrated that carbofuran does not affect the eclosion of *M. incognita* J_2. However, J_2 from eggs exposed to carbofuran lose great part of its infective capability (Figure 2).

Kanchiswamy *et al*., (2015) suggested that bioprospecting research on VOCs must not restrict only to laboratory studies, once such conditions are not able to precisely represent the real field conditions. As a consequence, experiments under greenhouse conditions were also performed and the results suggested the existence of practical potential for the use of compound **1**, as an isolated compound. However, the use of other VOCs produced by *F.o-21* has not to be rejected, once changes in concentrations, the dissolving vehicle, the applied quantities and the application frequency may increase the efficiency of the compounds to control PPN. Besides that, it is not to dismiss the possibility of a synergistic effect between these VOCs, once in nature they are produced as a whole by the fungus.

Microbial origin VOCs are commonly referred as less environmentally toxic antimicrobial compounds. In that manner, they are considered as an alternative to molecules currently used in agriculture. Nevertheless, studies concerning the target sites of such compounds in plant pathogens are still scarce. In the present work target sites from VOCs on J_2 were not studied, but J_2 exposed to compounds **1**, **2**, **7** and **8** were observed in a straight position and with internal vacuolization (data not showed), similar observations were reported after exposure of *M. incognita* J_2 to 4-methylthiobutyl isothiocyanate by Aissani *et al*., 2015.

5 CONCLUSIONS

Bioprospecting among thousands of VOCs produced by microorganisms may result in some new molecules exhibiting nematicidal activity and potentially new commercial products may be developed. Yet, this is an arduous task that begins in the laboratory and must mandatorily be extended to the open environment, where the nematode's J_2 and eggs must be challenged. Thus, this work demonstrates the nematicidal activity from diverse molecules produced by *Fo-21*, and the differential sensibility of J_2 and eggs to VOCs, besides showing the potential use of compound **1** in the field, after improvements in the application technology of this compound.

6 REFERENCES

Amaral, D. R., Oliveira, F. E. R., Oliveira D. F. (2003). Purification of two substances from bulbs of onion with nematicidal activity against *Meloidogyne exigua* Goeldi. Nematology, (6):859-864.

Aissani, N., Urgeghe, P. P., Oplos, C., Saba, M., Tocco, G., Petretto, G. L., Eloh, K., Menkissoglu-Spiroudi, U., Ntalli, N., Caboni, P., (2015). Nematicidal activity of volatilome of *Eruca sativa* on *Meloidogyne incognita*. Journal of Agricultural and Food Chemistry. (63):6120-6125.

Bitas, V., McCartney, N., Li, N., Demers, J., Kim, J-E., Kim, H-S., Brown, K. M., Kang, S., (2015). *Fusarium Oxysporum* Volatiles Enhance Plant Growth Via Affecting Auxin Transportand Signaling. Frontiers in Microbiology. (6):1248. doi: 10.3389/fmicb.2015.01248.

Barros, A.F., Campos, V.P., Silva, J.C.P., Pedroso, M.P., Medeiros, F.H.V., Pozza, E.A., Reale, A.L., (2014a). Nematicidal activity of volatile organic compounds emitted by *Brassica jucea, Azadirachta indica, Canavalia ensiformis, Mucuna pruriens* and *Cajanus cajan* against *Meloidogyne incognita.* Applied Soil Ecology. (80):34-43.

Barros, A.F., Campos, V.P., Silva, J.C.P., López, L.E., Silva, A.P., Pozza, E.A., Pedroso, L.A., (2014b). Tempo de exposição de juvenis de

segundo estádio a voláteis emitidos por macerados de nim e de mostarda e biofumigação contra *Meloidogyne incognita.* Nematropica, (44):190-199.

Busko, M., Kulik, T., Ostrowska, A., Goral, T., Perkowski, J., (2014). Quantitative volatile compound profiles in fungal cultures of three different *Fusarium graminearum* chemotypes. FEMS Microbiol. Lett, (359):85-93.

Campos, V.P., Pinho, R.S.C., Freire, E.S., (2010). Volatiles produced by interacting microorganisms potentially useful for the control of plant pathogens. Ciência e agrotecnologia, (3):525-535.

Chen, S.Y., Dickson, D.W., (2000). A technique for determining live second-stage juveniles of *Heterodera glycines*. Journal of Nematology, (32):117-121.

Cho, S.M., Kang, B.R., Han, S.H., Anderson, A.J., Park, J., Lee, Y., Cho, B.H., Yang, K., Ryu, C., Kim, Y.C., (2008). 2R, 3R-Butanediol, a Bacterial Volatile Produced by *Pseudomonas chlororaphis* O6, Is Involved in Induction of Systemic Tolerance to Drought in *Arabidopsis thaliana*. Mol Plant-Microbe Interact, (21):1067–1075.

Cortes-Barco, A.M., Goodwin, P.H., Hsiang, T., (2010). Comparison of induced resistance activated by benzothiadiazole, (2R,3R)-butanediol and an isoparaffin mixture against anthracnose of *Nicotiana benthamiana*. Plant Pathology, (59): 643-653.

Costa, L.S.A.S., Campos, V.P., Terra, W.C., Pfenning, L.H., (2015). Microbiota from *Meloidogyne exigua* egg masses and evidence for the effect of volatiles on infective juvenile survival. Nematology, (17):715–724.

Effmert, U., Kalderás, J., Warnke, R., Piechulla, B., (2012). Volatile Mediated Interactions Between Bacteria and Fungi in the Soil. J Chem. Ecol, (38):665–703.

Eisenback, J, D., Hunt, D.J., (2009). General Morphology. In Perry, R.N., Moens, M., Starr, F.J., Eds. 2009. Root-Knot Nematodes. CAB International, Wallingford, UK. p.23.

Fernando, W.G.D., Ramarathnam, R., Krishnamoorthy, A.S., Savchuk, S.C., (2005). Identification and use of potential bacterial organic antifungal volatiles in biocontrol. Soil Biology & Biochemistry, (37):955-964.

Freire, E.S., Campos, V.P., Oliveira, D.F., Pohlit, A.M., Norberto, N.P., Faria, M.R., (2012). Volatile substances on the antagonism between fungi, bacteria and *Meloidogyne incognita* and potentially fungi for nematode control. Journal of Nematology. (44):321-328.

Grimme, E., Zidack, N.K., Sikora, R.A., Strobel, G.A., Jacobsen, B.J., 2007. Comparison of *Muscodor albus* Volatiles with a Biorational Mixture for Control of Seedling Diseases of Sugar Beet and Root-Knot Nematode on Tomato. Plant Disease, (91):220-225.

Goverse, A., Smant, G. (2014). The activation and suppression of plant innate immunity by parasitic nematodes, (52):243-265.

Gu, Y.i., Mo, M.H., Zhou, J., Zou, C.S., Zhang, K.Q., (2007). Evaluation and identification of potential organic nematicidal volatiles from soil bacteria. Soil Biology & Biochemistry, (39):2567–2575.

Hussey, R.S., Barker, K.R., (1973). A comparison of methods for collecting inocula of *Meloidogyne* spp., including a new technique. Plant Disease Report, (57):1025-1028.

Kanchiswamy, C.N., Malnoy, M., Maffei, M.E., (2015). Chemical diversity of microbial volatiles and their potential for plant growth and productivity. Frontiers in Plant Science, (6):1-23.

Lemfack, M.C., Nickel, J., Dunkel, M., Preissner, R., Piechulla, B., (2014). mVOC: a database of microbial volatiles. Nucleic Acids Research, (42):744–748.

Li, M., Wen, F., Ying, H., He, M.M., Qin, X.J., Qing, D.Y., Huang, F.D., Hui, Y.S., (2012). A strategy to discover potential nematicidal fumigants based on toxic volatiles from nematicidal bacteria**.** African Journal of Microbiology Research, (6):6106-6113.

McCarter JP., (2008). Molecular Approaches Toward Resistance to Plant-Parasitic Nematodes**.** In: Berg, R. H and Taylor, C. G (eds) Cell Biology of Plant Nematode Parasitic. Plant Cell Monographs. 239-267.

Noling, J.W., (2002). The Practical Realities of Alternatives to Methyl Bromide: Concluding Remarks**.** Phytopathology. 92, 1373-5.

Payan, L.A., Jonhson, A.W., Littrell, R.H., (1987) Effects of nematicides and herbicides alone or combined on *Meloidogyne incognita* egg hatch and development. Annals of Applied Nematology, (1):67-70.

Seo, S. M., Kim, J., Koh, S. H., Ahn, Y. J., Park, I. K. (2014). Nematicidal activity of natural ester compounds and their analogues against pine wood nematode, *Bursaphelenchus xylophilus*. Journal of Agricultural and Food Chemistry, (62):9103-9108.

Sigma-Aldrich. Online.http://www.sigmaaldrich.com/brazil.html. Assessed 15 November 2015.

Strobel, G.A., Dirkse, E., Sears, J., Markworth, C., (2001). Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. Microbiology, (147):2943–2950.

Terra, W.C., Campos, V.P., Pedroso, M.P., Costa, A,L., Freire, E,S., Pinto, I,P., Silva, J.C.P., Lopez, L.E., (2016). Volatile molecules of *Fusarium oxysporum* are retained in water and control *Meloidogyne incognita*. In press.

Zhang, Q., Yang, L., Zhang, J., Wu, M., Chen, W., Jiang, D., Li, G. (2015). Production of anti-fungal volatiles by non-pathogenic *Fusarium oxysporum* and its efficacy in suppression of Verticillium wilt of cotton. Plant and Soil, (392):101-114.

http://bioinformatics.charite.de/movc/. Assessed 30 june 2016