LILIAN SIMARA ABREU SOARES COSTA

VOLATILES PRODUCED BY MICROBIOTA FROM *MELOIDOGYNE EXIGUA* EGG MASSES AND PLANT VOLATILE EMISSION IN RESPONSE TO SINGLE AND DUAL INFESTATIONS WITH SPIDER MITE AND NEMATODE

LAVRAS – MG

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

Orientador

Dr. Vicente Paulo Campos

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2014

Ficha Catalográfica Elaborada pela Coordenadoria de Produtos e Serviços da Biblioteca Universitária da UFLA

Costa, Lilian Simara Abreu Soares.
Volatiles produced by microbiota from *Meloidogyne exigua* egg masses and plant volatile emission in response to single and dual infestations with spider mite and nematode / Lilian Simara Abreu Soares Costa. – Lavras : UFLA, 2014.
94 p. : il.

Tese (doutorado) – Universidade Federal de Lavras, 2014.
Orientador: Vicente Paulo Campos.
Bibliografia.

1. Compostos orgânicos. 2. Defesa vegetal. 3. Controle biológico. I. Universidade Federal de Lavras. II. Título.
CDD – 632.96

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(VOLATEIS PRODUZIDOS PELA MICROBIOTA DAS MASSAS DE OVOS E DE PLANTAS EM RESPOSTA A INFESTAÇÕES SEPARADAS E CONJUNTAS COM ÁCAROS E NEMATOIDES)

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

APROVADA em 25 de julho de 2014.

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LAVRAS – MG

2014

AGRADECIMENTOS

A Deus, pela vida, pela proteção e por todas as suas bênçãos.

À Sta. Teresinha, cujo espírito gentil impregnou cada página deste capítulo.

Ao conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq, pela concessão da bolsa de estudos. À Universidade Federal de Lavras e Wageningen University-NE.

Ao Prof. Dr. Vicente Paulo Campos, pela orientação, conselhos e conhecimentos transmitidos, estímulo, amizade e exemplo de profissionalismo.

Aos professores do Departamento de Fitopatologia, pela formação acadêmica e apoio.

Ao funcionário Tarlei, por sua grande amizade e pelo valioso auxílio na avaliação dos trabalhos, pela paciência e bons conselhos e ao Cleber, pelos auxílios laboratoriais, pela convivência e amizade.

Aos colegas de trabalho, Eduardo, Willian, Aline, Julio, Lívia, Márcia, Arinaldo, Lilliana, pelo grande auxílio e dedicação durante a condução dos experimentos e pela valiosa amizade, que contribuíram para realização deste trabalho. Especialmente, aos "amigos, para os quais, qualquer hora é hora, seja no trabalho ou na descontração (colegas do curso de Mestrado/Doutorado). E aos amigos distantes, porém, amigos (Renata, Alex, Maria Clara e Davi, Hercules, Fernando, Fátima e José Mauro).

Em especial, aos meus novos velhos amigos de Wageningen-Holanda, Daniel, Cristiane, Renake, Vanessa, Juracy, Roberta, Vitor, Carlos, Wilson, Julio e Charles, serei eternamente grata por esse reencontro de almas.

Aos alunos da iniciação científica e estagiários do Laboratório de Nematologia, Guilherme, Marina, Luma, Felipe e Tatiane, obrigada pela amizade e convivência.

Aos demais colegas e funcionários do Departamento de Fitopatologia, pelo apoio e paciência, e aos funcionários responsáveis pela limpeza (Elenice), pela amizade, preocupação e descontração nas horas vagas.

A todos os meus familiares, aos meus irmãos Stella (Sophia e Gustavo), Lívia e Rinaldi em especial, aos meus pais Maria e Luiz Alberto, sem vocês, nada se realizaria.

Ao meu querido Peter, que me mostrou que realmente é possível viver um conto de Peter Pan e a "Terra do nunca" em uma mente barulhenta.

GENERAL ABSTRACT

Fungi and bacteria were found in coffee *Meloidogyne exigua* egg masses. In these masses, around 88% of the isolated fungi belonged to the *Fusarium oxysporum* species. Of the 69 bacteria isolates also obtained from these egg masses, the *Bacillus pumilus* and *B. methylotrophicus* prevailed. All obtained bacteria isolates caused 100% of mortality to *M. exigua* second stage juveniles (J2), when exposed to its volatile organic compounds (VOCs) for 3 days. *Pseudomonas putida* and *Microbacterium* sp caused very high mortality and, in 3 and 4 hours, respectively, killed 50 of the J2. On the other hand, the isolated fungi from the *M. exigua* egg masses were less effective in producing VOCs toxic to the J2 when compared to the bacteria. The *F. oxysporum* isolate 26 caused 95% of immobility and 27.2% of J2 mortality. *F. oxysporum* was present in coffee *M. exigua* egg masses during the entire year. The highest abundance of bacteria egg masses was verified during the summer period (III = Dec/Jan). The alterations in the levels of VOCs in the roots of cucumber plants after infestations performed separated and in association with *Tetranychus urticae* mites (S) and *M. incognita* J2 were always higher than those occurred in the leaves. In both organs, root and leaf, the altered compounds were terpenes, alkanes, aldehydes, phenolic compounds, with their levels represented below or above the control plant levels. The alterations in the levels of VOCs released by the roots of cucumber plants, after the infestation with J2, were detected by means of the Principal Component Analysis (PCA), only 9 and 13 days after inoculation. Most of the compounds found demonstrated higher concentration levels than those of the control plants. The predator of S (*Phytoseiulus permissilis*) showed strong attraction for plants infested by S, regardless of the presence or absence of the nematode in the same plant, by the use of the olfactometer. Nematode and spider mites alter the VOCs profile of cucumber plants, after infestation, which are detected by the natural enemy of the *Tetranychus urticae* mite.

Keywords: Volatile organic compounds. Egg masses. Biological control. Plant defense. Above-belowground interactions.

RESUMO GERAL

Foram encontrados fungos e bactérias em massas de ovos de *Meloidogyne exigua* do cafeeiro. Nas massas de ovos de *M. exigua,* cerca de 88% dos fungos isolados pertencem à espécie *Fusarium oxysporum*. Dos 69 isolados bacterianos também obtidos dessas massas de ovos prevaleceram as espécies *Bacillus pumilus* e *B.methylotrophicus*. Todos os isolados bacterianos obtidos causaram 100% de mortalidade à juvenil de segundo estágio (J2) de *M. exigua,* quando foram expostos por 3 dias aos seus compostos orgânicos voláteis (COVs). *Pseudomonas putida* e *Microbacterium* sp causaram mortalidade muito alta e, em 3 e 4 horas, respectivamente, mataram 50% dos J2. Por outro lado, os fungos isolados das massas de ovos de *M. exigua* foram menos eficazes na produção de COVs tóxicos a J2 comparados às bactérias. O isolado 26 de *F. oxysporum* causou 95% de imobilidade e 27,2% de mortalidade de J2. *F. oxysporum* e esteve presente nas massas de ovos de *M. exigua* do cafeeiro, durante todo o ano. Já, a maior abundância de bactérias nas massas de ovos foi constatada no período de verão (III = dez/jan). As alterações nos níveis de COVs nas raízes de plantas de pepino após infestações separadas e conjuntas com ácaros *Tetranychus urticae* (S) e J2 de *M. incognita* foram sempre maiores do que as alterações ocorridas nas folhas. Em ambos os órgãos, raiz e folha, os compostos alterados foram terpenos, alkanos, aldeídos, compostos fenólicos, sendo seus níveis representados abaixo ou acima dos níveis das plantas controle. As alterações nos níveis de COVs liberados pelas raízes de plantas de pepino, após infestação com J2, foram detectadas pela Análise de Componentes Principais (ACP), apenas 9 e 13 dias após inoculação. A maioria desses compostos encontrados demonstrou maiores níveis de concentração que as plantas controle. O predador (*Phytoseiulus permisilis*) demonstrou forte atração por plantas infestadas com S, independente da presença ou não do nematoide na mesma planta, pelo uso do olfactômetro. A presença do nematoide e do ácaro alteram o perfil de COVs de plantas de pepino, após a infestação, que são detectados pelo inimigo natural do ácaro *Tetranychus urticae*.

Palavras-chave: Compostos orgânicos voláteis. Massa de ovos. Controle biológico. Defesa de planta. Interações nas raízes e parte aérea.

SUMÁRIO

FIRST PART

1 INTRODUCTION

The soil environment is complex and having a changing biotic and abiotic aspect. Soil physical and biochemical compositions along with hits biology life are mostly involved in those changes (GEORGIEVA; CHRISTENSEN; STEVNBAK, 2005). Plant roots release substantial amounts of C- and N-containing compounds into the surrounding soil. Microorganisms are attracted to this nutritious environment and use the root exudates and lysates for growth and multiplication on the surface of root and in the adjacent rhizosphere soil (CHOUDHARY; JOHRI, 2009). The rhizosphere is a densely populated area in which the roots must compete with the invading root systems of neighboring plant species for space, water, and mineral nutrients, as well as with soilborn microorganisms, including bacteria, fungi, and feeding insects on abundant source of organic matter (PAPATHEODOROU et al., 2012). Thus, interaction of root – root, root – microbe, and root – insect establish communications which are likely to occur in this biologically active soil zone. However, due to the underground nature of plant roots, these intriguing interactions have largely been over-looked (WONDAFRASH; VAN DAM; TYTGAT, 2013).

Most plant parasitic nematodes are important soil pathogens on most food, horticultural and fiber crops. They cause yield losses and reduce food quality. Nematode management is difficult, complicated and costly especially with chemicals to maintain their populations below economic threshold levels. Biological suppression of plant parasitic nematodes with nematode predators, parasites or disease agents is a desirable alternative to chemicals. Emission of volatiles by bacteria and fungi has been known and reviewed by many authors

for a long time (CAMPOS; PINHO; FREIRE, 2010; KAI et al., 2009). Volatile production is species-specific and serve as (1) infochemicals for inter and intraorganismic communication, (2) cell-to-cell communication signals, (3) a possible carbon release valve, or (4) growth-promoting or inhibiting agents (KAI et al., 2009). The tropical soils are rich in biodiversity of beneficial microbes and the biocontrol potential of the resident microbial fauna and flora is quite unexploited. The structural composition of the bacterial microbiota of *M. exigua* egg masses remains unknown, as does the toxicity of volatile organic compounds (VOCs) produced by the fungi and bacteria that constitute the microbiota of the egg masses of this nematode. Then VOCs emittion may occur in these belowground interactions among nematode and microbiota leading to biological control in egg masses.

Plant volatile substances promote communication and interaction with the surrounding environment (DUDAREVA et al., 2006). The vast majority of studies examining the efflux of VOCs from terrestrial ecosystems have focused on the production of such substances by plants, which, according to Knudsen and Gershenzon (2006), can produce more than 1700 VOCs. Plants release about 1% of the secondary metabolites as VOCs into the atmosphere and from roots into the soil. In addition nematode infestation in plants may affect their VOCs emittions. The role of volatiles of microbial origin as signal molecules for plant defense has come to light recently. A comparison has been drawn between herbivores-induced plant volatiles (HIPV's) as an elicit or of plant defenses and two other classes of signaling molecules, C6 green-leaf volatile s (GLVs) and C4 bacterial volatiles which appear to prime plant defenses thereby enhancing the capacity to mobilize cellular defense responses when plants are faced with herbivore/pathogen attacks (DICKE; VAN LOON; SOLER, 2009). Plant responses on interaction among diversified organisms (insects, nematodes, root pathogen and symbiotic organisms) between above-and belowground and they

are known to be complex. However, little to nothing is known about the consequences of the changes on the plant volatile emissions in above-belowground interaction between nematodes and insect herbivores.

This inconsistency emphasizes the need to develop a greater understanding of the interactions between nematodes and the various other biotic components of the system. Therefore, in this study, we aimed to analyze the profile of volatile compounds emitted by cucumber plants in above-belowground interaction with the nematode *Meloidogyne incognita* and the two-spotted spider mite, *Tetranychus urticae* and the structure and involvement of egg mass microbiota on *M. exigua* in the coffee field.

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SECOND PART – ARTICLES

ARTICLE 1 Bacteria and fungi in egg masses of the coffee pathogen *Meloidogyne exigua* produce volatiles toxic to the second-stage juveniles

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Artigo preparado de acordo com as normas do periódico *Crop Science.*

ABSTRACT

The *Meloidogyne* egg mass colonization by fungi and bacteria is possible to occur and leave toxic substances to egg embryo and juveniles of this pathogen, at the site of colonization. We investigated the incidence of fungi and bacteria in egg masses of the coffee pathogen *Meloidogyne exigua* and the toxic effect of their volatile organic compounds (VOCs) on *M. exigua* second-stage juvenile (J2). Approximately 88% of the fungi isolated from *M. exigua* egg masses belong to the species *Fusarium oxysporum*. Of the 69 bacterial isolates obtained from *M. exigua* egg masses, the species *Bacillus pumilus* and *B. methylotrophicus* predominated. All the bacterial isolates caused 100% mortality to the J2s of *M. exigua* when they were exposed to the bacterial VOCs for three days. However, 50% of the bacterial isolates caused medium to very high mortality when the J2s were exposed to the bacterial VOCs for 24 hours only. *Pseudomonas putida* and *Microbacterium* sp. caused very high mortality to J2s and killed 50% of them in 3 and 4 hours, respectively. Conversely, the fungi isolated from *M. exigua* egg masses were less efficient in producing VOCs that are toxic to J2 compared to the bacterial isolates. However, the *F. oxysporum* isolate 26 caused 95% immobility and 27.2% mortality of J2. *F. oxysporum* was present in the *M. exigua* egg masses throughout the whole year, and the highest bacterial abundance was observed in the summer (December/January sampling). The data obtained in the present study indicate the continued colonization of fungi and bacteria in *M. exigua* egg masses. In addition, according to

the fungal and bacterial species present in the *M. exigua* egg masses, their VOCs can reduce the viable inoculum of *M. exigua* in coffee roots and may thus become biocontrol agents in certain farms.

Keywords: biological control, egg mass, parasitism, microbiota, volatiles organic compounds.

Introduction

Coffee is recognized as an agricultural product consumed worldwide. Half of the production demanded by the world coffee market originates from Brazil (Conab, 2014). In both Brazil and other Latin American countries that produce coffee, diseases caused by nematodes result in extensive losses to the coffee producers, especially the diseases caused by *Meloidogyne exigua* since the description of this species in 1887 (Campos and Villain, 2005). *Meloidogyne exigua* is highly spread among the coffee plantations in Brazil. In coffee plantations in southern Minas Gerais state, Brazil, where approximately one-fourth of all Brazilian coffee is grown, *M. exigua* has been reported in 22% of coffee plantations (Castro et al., 2008)

Field inoculum of *M. exigua* has commonly been used for research, avoiding the need for maintenance in greenhouse-inoculated

coffee plants. In the last five years, *M. exigua* has become scarce in a coffee plantation commonly sampled for inoculum supplies for our studies, which also exhibits rapid darkening of the root galls. Coffee galls of recently produced *M. exigua* are white and subsequently become dark cream. Once produced, the galls do not outgrow or coalesce, and the root segment decays. In the galls stays the female that lay half of the eggs and remain agglutinated by the matrix called egg mass around the vulva becoming inoculum promptly for spreading the pathogen (Campos and Villain, 2005). Mayol and Bergeson (1970) demonstrated that in sterilized conditions, *Meloidogyne* galls and roots are free of necrosis. In addition, in the same above-mentioned coffee field inoculum supplier, a lower J2 hatching percentage in the higher temperature periods of the year was observed by Costa et al. (2012) leading us to suspect microorganism involvement. How coffee root gall microbiota damage *M. exigua* remains unknown. Furthermore, the relationship of the coffee root gall color to the presence of microbiota is not well understood. Also unknown is the microbiota structure and how molecules released within the galls cause *M. exigua* damage. The *M. exigua* egg mass generally occurs underneath the coffee root epidermis, which may become a food source to maintain fungal and bacterial colonies in the coffee rhizosphere. Furthermore, the antagonistic fungi and bacteria to *M. exigua*, once found in the egg mass, might be a potential biological control agent to be used by the industry (Freire et al., 2012).

The perennial nature of the coffee crop (Vieria, 2008) allows the stability of ecological factors and the adaptation of microorganisms in the coffee rhizosphere for many consecutive years. Studies of fungal and

bacterial filtrates have demonstrated the existence of molecules toxic to *Meloidogyne* spp. in the filtrates (Oliveira et al., 2007; Oliveira et al., 2009). Therefore, even without parasitizing the nematodes, the final metabolic products of the microbiota present in the egg mass may cause damage to the different stages of these organisms.

In addition to liquid soluble molecules, the bacteria and fungi of soil and the plant rhizosphere emit volatile organic compounds (VOCs) that are toxic to nematodes (Fernando et al., 2005; Gu et al., 2007; Zou et al., 2007; Freire et al., 2012) and fungi, causing fungistasis (Fravel, 1988; Burgess et al., 1999; Campos et al., 2010), and may even act as long-range infochemicals (Kai et al., 2009). Moreover, VOCs emitted by bacteria are toxic to fungi that act as biocontrol agents (*Arthrobotrys conoides*, *Paecilomyces lilacinus*, *Pochonia chlamydosporia*) and to plant pathogenic fungi such as *Sclerotinia sclerotiorum* (Fernando et al., 2005; Campos et al., 2010). However, studies on the compounds produced by bacteria and fungi isolated from the structure of plant pathogenic nematodes, such as the egg masses of *Meloidogyne* spp, particularly the coffee root-knot nematode *M. exigua*, are scarce.

The incidence of fungi in egg masses of *Meloidogyne* sp. has been studied in the search for predators and parasites of eggs (Oka et al., 1997; Sharon et al., 1993; Kok et al., 2001). However, there are no studies about other fungi in the egg masses of the coffee root-knot *M. exigua*. The incidence of bacteria in *Meloidogyne* spp. egg masses is also largely unknown. Only the studies of Kok et al. (2001) and Papert et al. (2004) surveyed the bacterial species present in *Meloidogyne fallax* and *M. hapla* egg masses, but the authors did not analyze the production of volatiles by

the bacteria. The structural composition of the bacterial microbiota of *M. exigua* egg masses remains unknown, as does the toxicity of VOCs produced by the fungi and bacteria that constitute the microbiota of the egg masses of this nematode. Thus, this study aimed to accomplish the following: 1) to characterize the bacterial and fungal species in the egg masses of the coffee root-knot nematode *M. exigua*; 2) to study the abundance of bacteria and fungi in coffee galls during different periods of the year; and 3) to test the toxicity of VOCs emitted by the fungal and bacterial species found in *M. exigua* egg masses to the second-stage juvenile of the coffee pathogen *Meloidogyne exigua*.

Materials and Methods:

1. Sampling.

The coffee plantations chosen for sampling are located in the municipalities of Lavras and Varginha, state of Minas Gerais, Brazil, and have supplied *Meloidogyne exigua* inoculum for laboratory and greenhouse research. However, the extraction of *M. exigua* eggs from root samples of the coffee plants of these locations has declined in the last five years. Four samples containing thin roots infested by *M. exigua* were collected from the canopy of three coffee plants at a depth of 0-20 cm in the coffee plantations. The samples were collected in four periods representing the seasons of the year: winter (period I) sampling in June and July; spring (period II) sampling in September and October; summer (period III) sampling in December and January; and autumn (period IV)

sampling in March and April. The samples were stored at 8-10 °C for up to three days, after which the fungal and bacterial isolations were performed.

2. Bacterial isolation and estimates of the bacterial colonies formed from the egg masses.

These studies were conducted in two coffee plantations infested by *M. exigua* in two distinct locations (municipalities of Lavras and Varginha) and in four periods of the year, as described in the section above. Coffee root segments with galls were immersed in 70% alcohol for 30 seconds. Subsequently, the root segments were transferred to 0.1% hypochlorite for one minute and washed in distilled water. The egg masses (internal for new galls and external for old galls) were examined under a stereoscopic microscope, and 20 of the egg masses exhibiting darkening, which is an indicative symptom of microbiological parasitism as recommended by Stirling and Mankau (1979), were selected. For comparison purposes, 20 egg masses from new clear-colored galls were also collected. The 20 egg masses from both dark and clear galls were used separately to obtain microbial suspensions. To do so, the egg masses were washed three times in sterile saline (8.5 g $NaCl.1^{-1}$) and dissected at low temperature with the aid of a glass rod. Aliquots of 0.1 ml were obtained from these suspensions and were serially diluted by plating them in tryptic soy agar (TSA) medium with 50 $mg.1^{-1}$ cycloheximide fungicide. Three replicates were used for each dilution, and the population density was expressed as colony forming units (CFU) to

evaluate the bacterial abundance in *M. exigua* egg masses. Six days after the replications were made, all the bacterial colonies formed were counted. The bacterial colonies with different morphologies by visual inspection were transferred to new medium. Only the pure cultures were stored at 150 g. 1^{-1} glycerol at -80°C and used in the assays of volatile organic compounds (VOCs) to evaluate the mortality and immobility of the second-stage juvenile (J2) of *M. exigua*.

3. Fungal isolation from egg masses.

Using the same procedure described above, 20 egg masses were obtained from dark-colored galls and 20 from clear-colored galls, after which surface sterilization was performed. The egg masses were dissected separately in sterile saline (8.5 g $NaCl.1^{-1}$) in a sterile well slide. The contents of two of the dissected egg masses were plated on malt medium (MA) containing 50 $mg.1^{-1}$of the streptomycin antibiotic to prevent bacterial growth. The Petri dishes were incubated at 25°C in the dark. After fungal growth, the colonies visually recognized as distinct were transferred to another dish containing MA because the only interest was to assess the species diversity. The isolates were preserved by the method of Castellani (1939) until they were used in subsequent assays.

4. Characterization of the genus and species of fungi and bacteria isolated from the egg masses.

The fungal isolates obtained from egg masses of *M. exigua* were classified at the level of genus and species through the observation of the morphological characteristics of conidia, hyphae and conidiophores, with the aid of identification keys.

The bacterial isolates were classified by PCR using specific primers after DNA extraction, quantification and amplification of the16S rRNA region. The DNA was extracted after transferring the stock culture to TSA and 523 (Kado and Heskett, 1970).

5. Fungal colonization in the root segments of infested coffee plants.

Root segments with dark and clear galls were randomly obtained from the coffee plantations of Lavras and Varginha, state of Minas Gerais, Brazil. Approximately four 5-mm segments consisting almost entirely of the gall caused by the nematode were surface sterilized for each sampling period (I = June/July, II = September/October, III = December/January and IV = March/April). These gall segments were distributed equidistantly at four points in a Petri dish with water-agar (WA) medium, and each replicate consisted of two dishes. The Petri dishes were randomly distributed in incubators and maintained at 28°C. Forty-eight hours after incubation, the Petri dishes were examined under a stereoscopic microscope to check for fungal growth from each gall segment in the Petri dish. Of the eight segments evaluated per replicate,

the percentage of fungal incidence by replicate (two Petri dishes) was calculated. After the assessment, the Petri dishes were returned to the same incubators, where they were maintained for three more days. Morphology, color, spores and mycelial structures were observed in the colonies that developed in the Petri dishes to characterize the species colonizing the root segments.

6. Mortality of Meloidogyne exigua second-stage juveniles exposed to volatile organic compounds produced by fungi.

The nematicidal activity of VOCs of fungi isolated from egg masses was evaluated according to the methodology of Fernando et al. (2005) with some modifications. The J2s used in the assays were obtained from eggs of coffee galls extracted according to Hussey and Barker (1973) and placed in a hatching chamber formed by a mesh screen and a glass funnel. Only the J2s obtained from the second day onwards were used in the assays. Each fungus was plated in one of the Petri dish compartments containing MA. When the fungal colony reached 4.5 cm in diameter, an aqueous suspension containing approximately 100 J2s of *M. exigua* was poured to the adjacent compartment in the Petri dish onto a WA layer. As a control, the same amount of MA with no fungi was added to one of the compartments of the Petri dishes, and the J2s were added to the adjacent compartment as previously described. The Petri dishes were sealed with plastic wrap to prevent the escape of VOCs and incubated at 25°C in the dark for 72 hours. Then, the mobile and immobile J2s were estimated under a microscope with an inverted objective. The immobile J2s were

transferred to sterile distilled water, and those still immobile after 24 hours were considered dead (mortality).

The nematicidal activity (NA) was calculated using the formula NA = IN/TN x 100, in which IN represented the number of immobile or dead nematodes and TN represents the total number of J2s.

7. Mortality of Meloidogyne exigua second-stage juveniles exposed to volatile organic compounds produced by bacteria.

The bacteria isolated from the egg masses and kept in the freezer at -80 ºC were grown in Petri dishes containing TSA medium and incubated at 28 ºC for 24 hours. Subsequently, the bacterial isolates were transferred to TSA medium placed in one of the compartments of a split Petri dish. In the adjacent compartment, a layer of WA medium was placed, and a suspension with 100 J2s of *M. exigua* was added to the WA medium as described by Fernando et al. (2005). Specimens of *M. exigua* obtained from field coffee plantations and from coffee plants kept in a greenhouse were used for these in vitro assays. As a control, the same amount of TSA medium with no bacteria was added to one of the Petri dish compartments, and the J2s were added to the adjacent compartment on the WA medium. The Petri dishes were sealed with plastic wrap to prevent the escape of VOCs and incubated at 28 °C in the dark. The J2 immobility and mortality were estimated after 24, 48 and 72 hours.

***8. Exposure time of Meloidogyne exigua second-stage juvenile to the volatile organic compounds produced by Pseudomonas putida and Microbacterium* sp.**

Pseudomonas putida and *Microbacterium* sp. isolates were used in this assay because they emitted VOCs highly toxic to J2s after 24 hours of exposure. The bacterial isolates were transferred to TSA medium and placed in one of the Petri dish compartments. In the adjacent compartment, a layer of the WA medium was added, over which was placed a suspension with 100 J2s of *M. exigua*. As a control, the same amount of the TSA medium with no bacteria was added to one of the Petri dish compartments, and the J2s were added to the adjacent compartment on the WA medium. The Petri dishes were sealed with plastic wrap to prevent the escape of VOCs and incubated at 28 °C in the dark. Four split Petri dishes were opened after 3, 6, 12 or 24 hours of sealing, and the mobile and immobile J2s were counted under a stereoscopic microscope. The immobile J2s were transferred to sterile distilled water, and those still immobile after 24 hours were considered dead (mortality).

9. Data analysis.

All of the assays were repeated twice using a completely randomized experimental design with four replicates per treatment. The data were subjected to the Shapiro-Wilk and Bartlett tests ($p > 0,05$) to confirm normal distribution and homoscedasticity, respectively, for

analysis of variance (ANOVA). Accepting these assumptions, the data were subjected to one-way or two-way ANOVA and treatments means were compared with the Scott and Knott (1974) and Tukey tests ($p< 0,05$). The regression model was fit to the quantitative variables.

Results

Microbiota of Meloidogyne exigua egg masses

The species *Fusarium oxysporum* predominated among the fungal isolates, corresponding to approximately 88% of the isolates obtained in the present study. Another *Fusarium* species, *F. solani,* occurred in two isolates (7% of total). Only one isolate was characterized as *Cylindrocarpon* sp. (Table 1).

Fifteen bacterial isolates identified by DNA with distinct morphology were obtained. The genus *Bacillus* predominated among the bacterial isolates, being present in 80% of the isolates and with higher incidences of *B. pumilus* and *B. methylotrophicus*. *Microbacterium* sp., *Pseudomonas putida* and *Enterobacter* sp., *Bacillus fusiformis* and *Bacillus* sp. (not identified) were also encountered in the *M. exigua* egg masses (Table 1).

Table 1. Genera and species of bacterial and fungal strains isolated from egg masses of *Meloidogyne exigua* (clear and dark galls) collected in the field soil from Lavras and Varginha towns, Minas Gerais State, Brazil.

Genera and Species of Bacteria	**Number of isolates**	**Seasons of collecting samples**	**Type of galls**	**Sampling places (towns)**
Bacillus pumilus	4	I, II	dark, clear	Lavras, Varginha
Bacillus methylotrophicus	6	I, II, III	dark, clear	Lavras, Varginha
Microbacterium sp.	1	I	Dark	Lavras
Pseudomonas putida	1	II	clear	Lavras
Enterobacter sp.	1	IV	clear	Lavras
Bacillus fusiformis	1	II	clear	Lavras
Bacillus sp.	1	II	clear	Varginha
Total	15	-	-	-
Genera and Species of Fungi	**Number of isolates**	**Seasons of collecting samples**	**Type of galls**	**Sampling places (towns)**
Cylindrocarpon sp.	1	IV	clear	Varginha
Fusarium oxysporum	23	I, II, III e IV	dark, clear	Lavras, Varginha
Fusarium solani	2	I, II III e IV	clear	Lavras, Varginha
Total	26	-	-	-

I = Winter (june/july); **II** = Spring (september/october); **III** = Summer (december/ january); **IV** = Autumn (march/april)

Isolation of fungal and bacterial species in root segments with dark and clear galls

Fungal and bacterial isolates were obtained in all sampling periods. The highest number of bacterial and fungal isolates was obtained in the period of September/January (periods II and III) and September/October (period II), respectively (Table 2).

Table 2. Number of isolates encountered in several time periods of the year (seasons) in field soil from Lavras and Varginha towns, Minas Gerais State, Brazil.

Seasons of collecting samples	**Nº of isolates**			
	VARGINHA		**LAVRAS**	
	Bacteria	**Fungi**	**Bacteria**	**Fungi**
I	9	2	8	7
II	10	3	14	3
III	12	3	3	3
IV	5	2	8	3
Total	36	10	33	16

I = Winter (june/july); **II** = Spring (september/october); **III** = Summer **(**december/ january); **IV** = Autumn (march/april)

The dark and clear galls provided approximately 65% and 35% of the fungal isolates, respectively. The distribution of the isolates during the sampling periods was almost uniform in the municipality of Varginha (state of Minas Gerais), whereas in the municipality of Lavras (state of Minas Gerais), a higher number of isolates was obtained in June/July

(period I). *F. oxysporum* was observed in all sampling periods in the egg masses of both dark and clear galls of the two coffee plantations studied.

Approximately 58% and 42% of the bacterial isolates were obtained in the egg masses of dark and clear galls, respectively. *B. pumilus* and *B. Methylotrophicus* were the most frequently observed among all the bacterial species found in both coffee plantations studied, occurring in egg masses of both dark and clear galls in the sampling periods of June/July (I) and September/October (II). However, *B. methylotrophicus* was also observed in December/January (III). Neither *B. pumilus* or *B. methylotrophicus* was observed in March/April (IV) (Table 1). Sixty-nine bacterial isolates were obtained in the first isolation and with a higher incidence in September/October (II) and December/January (III) (Table 2). However, approximately 54 of those isolates did not grow when transferred to the TSA and 523 medium, making it impossible to identify the species and to use them in later assays; therefore, 15 isolates were used in later assays (Table 1).

The bacterial abundance in *M. exigua* egg masses from coffee plants of the two studied locations, expressed as colony forming units (CFUs), revealed a significant interaction between the periods of the year and the type of galls (dark or clear) (Lavras: $F_{3,16} = 12{,}50$; $P < 0{,}001$) (Varginha: $F_{3,16} = 12{,}26$; $P < 0{,}001$). The total CFU count for all the sampling periods was higher in egg masses of dark galls than in egg masses of clear galls (Lavras: $F_{3,16} = 25{,}00$; $P < 0{,}001$). The period of the year with higher CFU counts in the egg masses from coffee plantations in the two studied locations was December/January (III) in both segments containing clear and dark galls, with CFU counts between $10^7$and 10^8 /20

egg masses. Conversely, a significant decrease in bacterial abundance (CFUs) occurred in root segments with clear galls collected in March/April (IV). Minor changes occurred in other sampling periods in the different galls segments (dark and clear) (Figure 1).

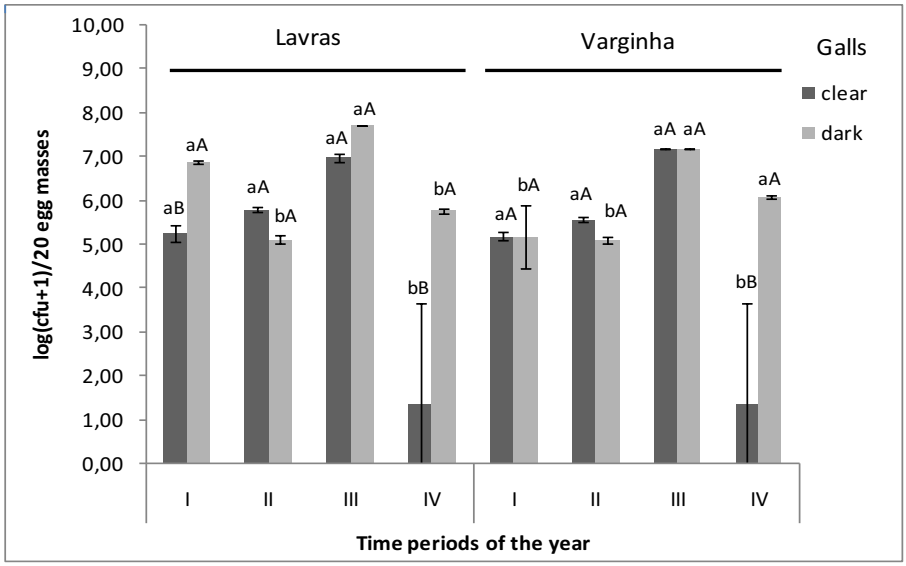

Figure 1: Colony forming units of bacteria (CFU) from coffee root egg masses (clear and dark galls) collected in the field of Lavras and Varginha towns, Minas Gerais State, Brazil. **I** = Winter (june/july); **II** = Spring (september/october); **III** = Summer **(**december/ january); **IV** = Autumn (march/april). Analysis by two way ANOVA. Means followed by same letter (small letters for comparison within time periods, and capital letters for comparison between clear and dark galls) are not significantly different according to the Tukey test ($P< 0.05$). The bars indicate the mean standard errors.

Fungal colonization in root segments with dark and clear galls

The evaluation of the surface colonization of gall segments by fungi did not exhibit significant interactions between sampling periods (I,

II, III and IV) and the type of gall (dark or clear) in the coffee plantation of the municipality of Varginha ($F_{3,24} = 0,60$; $P = 0,621$) . In dismembered analysis by one way ANOVA, the colonization of root segments with dark galls was significantly higher than the colonization of root segments with clear galls ($F_{3,24} = 10,80$; $P < 0,005$) (Table 3), confirming the higher fungal incidence in the egg masses of dark gall segments at the time of the isolation procedure (Table 1). However, the surface colonization by fungi was higher ($F_{3,24} = 5,40$; $P = 0,005$) in root segments collected in September/October (II) and December/January (III) than in the winter period (June/July) (Table 3).

Table 3. Mean ± SE fungal colonization segments (%) of coffee roots (clear and dark galls) from Varginha farm (Brazil) during different time periods of the year (seasons).

Seasons of collecting samples	% Fungal Colonization
I = Winter (june/july)	65.62 ± 9.37b
II = Spring (september/october)	90.62 ± 4.57a
III = Summer **(**december/ january)	93.75 ± 4.09a
IV= Autumn (march/may)	75.00 ± 6.68ab
F = 5,40 *P* = 0,005	
Type of galls	
clear	71,87 ± 5.03b
dark	90,62 ± 4.49a
F = 10,80 *P* <0,005	

Analysis by one way ANOVA. Means followed by different letters were significantly different in the column according to the Tukey test ($P < 0.05$).

In the coffee plantation of the municipality of Lavras, Minas Gerais, the surface colonization of the gall segments by fungi revealed significant interactions between the sampling periods (I, II, III and IV) and the type of galls (dark or clear) ($F_{3,24} = 6,55$; $P< 0,005$). In this coffee plantation, the percentage of fungal colonization in the dark gall segments was 100% at all sampling periods, with values greater than or equal to the growth in those segments with clear galls (Figure 2). This result also confirms the higher fungal incidence in the egg masses of segments with dark galls compared with segments with clear galls at the time of the isolation process ($F_{1,24} = 16,33$; $P< 0,001$). A significant decrease in fungal growth occurred only in segments with clear galls in September/October (II) compared with the fungi growth in segments with dark galls. The variation in the percentage colonization in the root segments with dark galls was not significant between the four sampling periods (I, II, III and IV) ($F_{3,24} = 0,00$; $P> 0,005$), although the variation was high (Figure 2).

The fungal colonies observed in gall segments always exhibited the same morphology and color. In morphological analysis of fungus growth of the gall segments, *Fusarium oxysporum* was always observed, and this species also predominated in the isolates of *M. exigua* egg masses (Table 1).

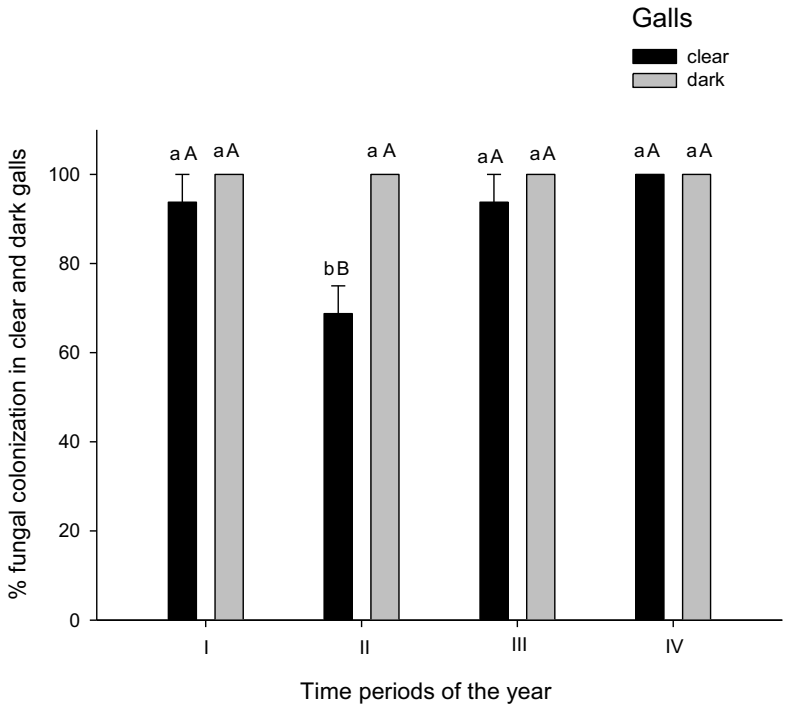

Figure 2: Fungal colonization segments (%) of coffee roots (clear and dark galls) from Lavras farm, (Brazil). Treatment labels: **I** = Winter (june/july); **II** = Spring (september/october); **III** = Summer **(**december/ january); **IV** = Autumn (march/may). Analysis by two way ANOVA.Means followed by same letter (small letters for comparison within time periods, and capital letters for comparison between clear and dark galls) are not significantly different according to the Tukey test ($P< 0.05$). The bars indicate the mean standard errors.

Nematicidal activity of volatile organic compounds emitted by fungi

Emitted VOCs from the fungi isolated from the egg masses caused variable toxicity to J2s of *M. exigua* after 72 hours of exposure. The percentage mortality (M) and immobility (IM) was separated into three and four groups, respectively, by the Scott-Knott statistical test. The immobility levels were defined as follows: a = no effect, 0.00≤IM≤12.97; b= low effect, 15.15≤IM≤18.00; c = medium, 24.42; and d = high, IM 94.92. The mortality levels were as follows: a = no effect, 1.40≤M≤12.27; b = low effect, 12.97≤M≤18.00; and c = medium, 27.32.Thus, only *F. oxysporum* isolates caused low, medium and high immobility. *F. oxysporum* isolates also caused low and medium mortality, especially the *F. oxysporum* isolate 26, which emitted VOCs that caused high immobility and medium mortality of the J2 of *M. exigua* (Table 5).

Table 5. Mean ± SE percentage of immobility and mortality of second stage juveniles of *Meloidogyne exigua* after 72 hours exposure to volatile organic compounds produced by fungi isolated from *M. exigua* egg masses.

Species of Fungi	Immobility (%)	Mortality (%)
Control	0.00 ± 0.00a	1.40 ± 1.40a
Cylindrocarpon sp-isol.1	5.32 ± 0.38a	5.32 ± 0.38a
F. oxysporum - isol.2	6.30 ± 4.70a	5.80 ± 3.36a
F. oxysporum - isol.3	7.17 ± 0.62a	6.30 ± 4.70a
F. oxysporum - isol.4	7.52 ± 1.14a	6.62 ± 2.44a
F. oxysporum - isol.5	7.97 ± 1.18a	7.17 ± 0.62a
F. oxysporum - isol.6	8.22 ± 1.13a	7.52 ± 1.14a
F. oxysporum - isol.7	8.52 ± 1.39a	7.97 ± 1.18a
F. oxysporum - isol.8	8.70 ± 1.22a	8.22 ± 1.13a
F. oxysporum - isol.9	8.85 ± 0.42a	8.52 ± 1.39a
F. oxysporum - isol.10	9.00 ± 1.18a	8.70 ± 1.22a
Fusarium solani - isol.11	9.05 ± 1.57a	8.85 ± 0.42a
F. oxysporum - isol.12	9.25 ± 1.10a	9.00 ± 1.18a
F. oxysporum - isol.13	9.80 ± 2.46a	9.25 ± 1.10a
F. oxysporum - isol.14	10.35 ± 1.26a	9.80 ± 2.46a
F. oxysporum - isol.15	10.77 ± 0.55a	10.35 ± 1.26a
Fusarium solani - isol.16	11.82 ± 1.77a	10.77 ± 0.55a
F. oxysporum - isol.17	12.27 ± 1.25a	11.60 ± 2.08a
F. oxysporum - isol.18	12.97 ± 0.61a	12.27 ± 1.25a
F. oxysporum - isol.19	15.15 ± 2.15b	12.97 ± 0.61b
F. oxysporum - isol.20	16.07 ± 1.52b	14.65 ± 1.34b
F. oxysporum - isol.21	16.12 ± 0.64b	15.15 ± 2.15b
F. oxysporum - isol.22	16.87 ± 5.38b	16.07 ± 1.52b
F. oxysporum - isol.23	17.75 ± 2.16b	16.12 ± 0.64b
F. oxysporum - isol.24	18.00 ± 1.98b	17.75 ± 2.16b
F. oxysporum - isol.25	24.42 ± 2.68c	18.00 ± 1.98b
F. oxysporum - isol.26	**94.92 ± 3.83d**	**27.32 ± 4.14c**
	($F_{26,81}$ = 66,6; *P*<0,001)	($F_{26,81}$ = 7,27; *P*<0,001)

Control: J2 stored in water.

Analysis by one way ANOVA. Means followed by same letter in the column are not significantly different according to the Scott and Knott's test at 5% probability.

Nematicidal activity of volatile organic compounds emitted by bacteria

The capacity to produce VOCs toxic to *M. exigua* in the first assay that contained 69 bacterial isolates was high after 72 hours of exposure of the J2s to the VOCs. All the isolates evaluated in this first assay caused 100% mortality of the J2s. Therefore, we decided to reduce the exposure time to 24 hours. However, from the culture replication of the 69 isolates obtained from *M. exigua* egg masses, approximately 77% did not grow in the artificial culture medium. Therefore, only 16 isolates could be used in the second assay; of these, 15 were identified to the genus and species level, but one isolate, which grew in MA in the second culture replication, was not identified (Table 2). The exposure of J2s for 24 hours demonstrated sensitivity of *M. exigua* to VOCs emitted by different bacterial isolates from *M. exigua* egg masses. The percentage mortality defined five groups that were separated by the Scott-Knott statistical test: a = very high, 71.78≤ M≤100; b = high, 34.89≤M≤48.77; c = medium, 21.86≤M≤28.68; d = low, 13.29; and e = no effect, i.e., equal to the control group, 2.70≤M≤7.96 (Figure 3).

The VOCs emitted by *Pseudomonas putida* isol. 11, *Microbacterium* sp. isol. 4, (unidentified) isol. 46, *B. metylotrophicus* isol. 56, *B. pumilus* isol. 9, *B.p*., isol.22 caused very high mortality in *M. exigua* J2. High J2 mortality was caused by the VOCs produced by *Enterobacter* sp. isol. 57, *B. pumilus* isol. 1 and *Bacillus* sp. isol. 29 in *M. exigua* J2. Medium mortality of the J2s of *M. exigua* was caused by *B. metylotrophicus* isol. 15, *B. pumilus* isol. 7. Low mortality was caused by VOCs produced only by *B. fusiformis* isol. 17. No effect was observed for

the VOCs produced by the bacteria *B. methylotrophicus* isolate 18, 8, 34 or 32 (Figure 3).

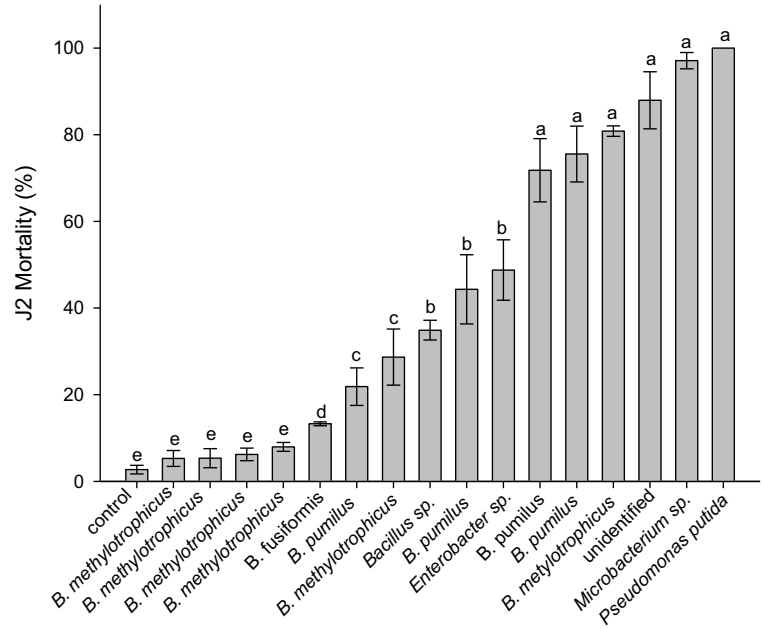

Figure 3 Percentage of mortality of second stage juveniles (J2) of *Meloidogyne exigua* after 24 hours exposure to volatile organic compounds produced by bacteria isolated from *M. exigua* egg masses. Means followed by same letter are not significantly different according to Scott and Knott's test ($P< 0.05$). The bars indicate the mean standard errors. Treatment labels: control; *B. methylotrophicus,*(*B.m.*) isol. 32; *B.m.*, isol. 34; *B.m.*, isol. 8; *B.m.*, isol. 18; *B. fusiformis*, isol. 17; *B. pumilus*, isol. 7; *B.m.*, isol. 15; *Bacillus* sp., isol. 29; *B. pumilus*, isol. 1; *Enterobacter* sp., isol. 57; *B.pumilus*, isol. 22; *B. pumilus*, isol. 9; *B.m.*, isol. 56; unidentified, isol. 46; *Microbacterium* sp., isol.4; *Pseudomonas putida*, isol. 11.

However, the question as to the best exposure time interval between 24 and 72 hours remained unanswered. To answer this question, the exposure of J2s to bacterial VOCs for 48 hours was evaluated. In this assay (third), the same isolates tested in the previous assay were used but with an exposure time of 48 hours. The following results were found: low mortality of J2s (±30%) by the VOCs of *B. methylotrophicus* isolate 15 (level c); high mortality of J2s ($90.25 \leq M \leq 95.22$) when exposed to VOCs emitted by *B. methylotrophicus* isolates 34 and 8, *Pseudomonas putida* and *Bacillus* sp. (level b); and very high mortality of J2s ($97.90 \leq M \leq 100$) (level a) caused by the VOCs produced by all remaining isolates (Figure 4). These results are similar to the ones obtained for an exposure time of 72 hours.

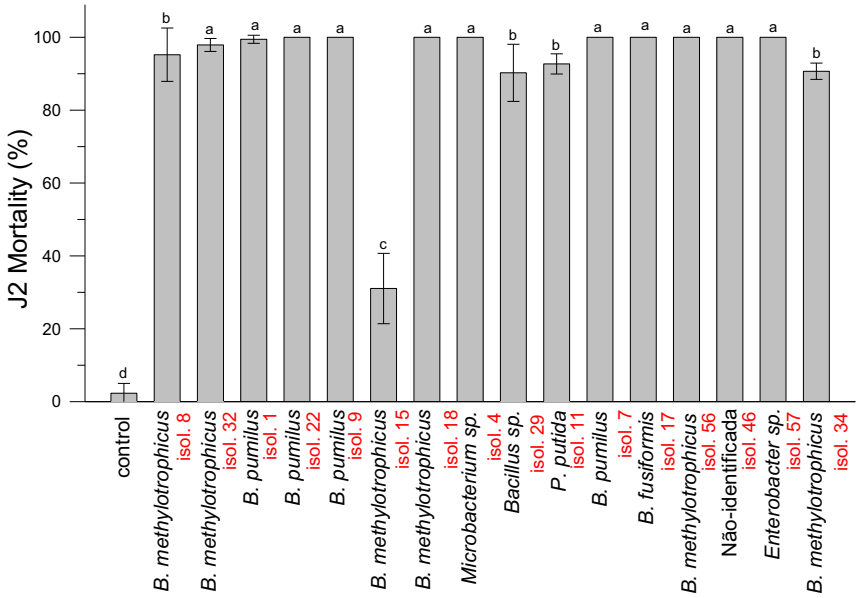

Figure 4 Percentage of mortality of second stage juveniles (J2) of *Meloidogyne exigua* after 48 hours exposure to volatile organic compounds produced by bacteria isolated from *M. exigua* egg masses. Means followed by same letter are not significantly different according to Scott and Knott's test at 5% probability.

Sensitivity of second-stage juveniles to bacterial VOCs

The exposure times of the *M. exigua* J2s to the VOCs produced after three hours of storage reflected the rate of VOC production by the bacteria *Microbacterium* sp. and *Pseudomonas putida* in a similar manner. For both bacterial species, the logarithmic phase of VOC production occurred between three and 12 hours, and the plateau was reached after 12 hours of exposure until the end of the assay. Therefore, 4 and 3 hours of exposure of J2s to the VOCs produced by *Microbacterium*

sp. and *Pseudomonas putida* respectively killed over 50% of the nematodes (Figure 5).

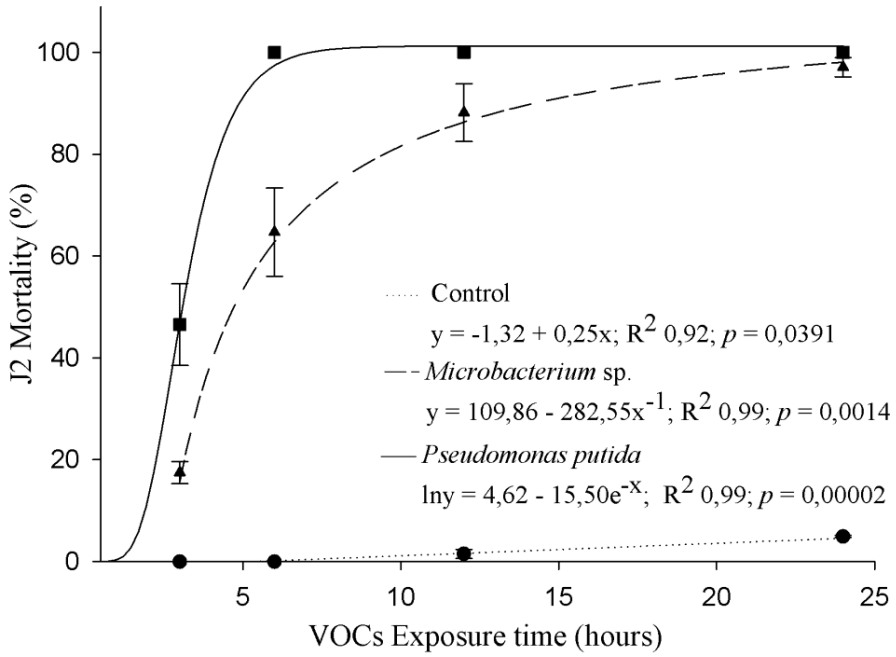

Figure 5 Mortality progress curve of second stage juveniles (J2) of *Meloidogyne exigua* after exposure to volatile organic compounds (VOCs) produced by *Microbacterium sp.* and *Pseudomonas putida*. The bars indicate the mean standard errors.

Discussion

In the present study, *Fusarium* species with a predominance of *F. oxysporum* were abundant in the egg masses of the coffee root-knot nematode *Meloidogyne exigua* (Table 1), which corroborates the study by Freire et al. (2012), who also found that species of the genus *Fusarium* were the most frequent species associated with *M. exigua* eggs and egg masses. Similarly, other researchers have found *F. oxysporum* and *F. solani* in the plant rhizosphere, soil and cysts (Crump et al., 1987; Meyer et al., 1990; Costa et al., 1997). In addition to these ecosystems, *F. oxysporum* has also been observed endophytically (Athman et al., 2006; Sikora et al., 2003). Moreover, several studies have demonstrated the biocontrol potential of non-pathogenic *F. oxysporum* in disease control (Dhingra et al., 2006; Nel et al., 2006). Other fungi have been found in the egg masses of the pathosystems *M. hapla* and tomato plant and *M. hapla* and potato, such as *Acremonium* sp., *Cylindrocarpon destructans*, *C. olidum*, *C. olidum*, *Mucorales*, and *Penicillium* sp. The fungi *Trichoderma harzianum*, *Cladosporium* sp., *Paecilomyces carneus*, and *Penicillium* sp. were found in *M. fallax* egg masses (Kok et al., 2001).

In many studies, the isolation of resident fungi in egg masses, including *Fusarium* species, has been neglected, with the studies focusing mostly on the search for the presence of predatory and parasitic fungi of both *Meloidogyne* spp. egg masses and the rhizosphere of infested plants (Barron, 1977; Gray, 1988; Kerry, 1984; Morgan-Jones and Rodriguez-Kabana, 1988).

The competitive ability of *F. oxysporum*in the *M. exigua* egg masses must be high because this fungus occurred in the highest number of the isolates (percentage) from *M. exigua* egg masses and was predominant in the colonization of the gall segments (Table 1). Freire et al. (2012) studied the antagonism of fungal species found in the coffee rhizosphere to *Arthrobotrys conoides* and only found antagonism with the *Fusarium* species. In the present study, the predominance of *F. oxysporum* throughout the whole year in the *M. exigua* egg masses demonstrates that the growth of *F. oxysporum* in the egg masses is independent of seasonal variations and that *F. oxysporum* must produce survival structures in cold and dry periods. Therefore, *F. oxysporum* may become a biological control agent even without being an egg parasite or J2 predator because it has been shown that *F. moniliforme* produces a non-volatile compound that is toxic to *M. exigua* (Amaral et al., 2003). Thus, future studies should assess the antagonistic potential of *F. oxysporum* isolates in competition with other coffee rhizosphere fungi and in the antagonism to *M. exigua*. However, *F. oxysporum* has been suggested to increase the severity of diseases caused by nematodes not only in coffee plants but also in other pathosystems (Bertrand et al., 2000). In addition, a higher frequency of DNA polymorphisms in *F. oxysporum* compared with other fungal species enables a greater chance of pathogenic adaptation in plants, which raises concerns of plant pathologists (Fourie et al., 2011).

Although bacteria are the most abundant in the microbiota of *M. exigua* egg mass, they remain understudied. Only Kok et al. (2001) and Papert et al. (2004), studied the structure of the bacterial microbiota of

the *Meloidogyne* species from temperate climates, such as *M. fallax* and *M. hapla,* and found 16 bacterial species. In the present study, *B. pumilus* and *B. methylotrophicus* exhibited the highest incidences in the *M. exigua* egg masses (Table 1). *Bacillus* species were also observed in *M. fallax* and *M. hapla* egg masses (Kok et al., 2001). *B. pumilus* also occurs in the rhizosphere of cucumber plants and exhibits high antagonist potential against *Rhizoctonia solani* in this plant (Huang et al., 2012). *B.methylotrophicus* isolate produces in culture filtrates the active substance 2-(phenylamino) propanoic acid, which strongly inhibits the *Magnaporthe oryzae* fungus in rice crops (Shan et al., 2013). The bacterial species found in the *M. exigua* egg masse, such as *Pseudomonas putida*, *Microbacterium* sp. and *Enterobacter* sp., have also been found in the rhizosphere of *Camellia sinensis*, maize and coconut, respectively (Sarkar et al., 2009; Sarkar et al., 2010; George et al., 2013; Gao et al., 2013). However, *B.fusiformis* has not been found colonizing the rhizosphere but instead in the soil as a biotransforming agent (Zhao et al., 2012) . Species of the genus *Bacillus* are being commercially exploited in the global market as biological agents for the control of nematodes and other plant pathogens (Davies, 2005; Campos et al., 2010).

The adaptation of bacterial strains to a specific substrate in nature, such as egg masses, makes the strains unable to effectively use artificial substrates for in vitro culture. Kok et al. (2001) observed loss of bacterial growth ability in culture replications. These authors reported a 22% loss of the growth ability of bacteria isolated from *M. fallax* egg masses of tomatoes plants, whereas in the present study, we observed approximately 77% loss of bacterial growth ability in culture replications. However, in

the present study, even with a reduced number of bacteria that tolerated multiple subcultures in artificial media, the number of bacterial species in the gelatinous matrix of *M. exigua* was diversified (Table 1).

In the present study, some of the fungal VOCs, especially those produced by *F. oxysporum* isolates, caused immobility and mortality, thereby revealing a nematicidal and nematostatic effect (Table 5). VOCs of *F. oxysporum* from the coffee rhizosphere in other locations also exhibit nematicidal effects on *M. incognita* (Freire et al., 2012). In addition, VOCs of other fungal species have been demonstrated to possess nematicidal and fungicidal effects (Campos et al., 2010), and the same has been observed for VOCs emitted by plants (Barros et al., 2014). However, a higher nematicidal effect was observed in the bacterial VOCs of *M. exigua* egg masses, causing high J2 mortality when they were exposed to VOCs for 24, 48 and 72 hours (Fig 3 and 4). Several researchers have used exposure times between 48 and 72 hours to evaluate the bacterial isolates' abilities to produce VOCs that are effective in the biocontrol of fungi and nematodes (Fernando et al., 2005; Gu et al., 2007; Zou et al., 2007). However, in the present study, 24 hours of exposure was sufficient to demonstrate differences in the production rate of VOCs by the bacteria isolated from *M. exigua* egg masses (Fig 3). It is important to highlight that isolates with high and very high nematicidal ability (mortality) in the assays of exposure to VOCs for 24 hours, such as *Microbacterium* sp., *Pseudomonas putida*, *B. pumilus* isolate 9 and 22, isolate 46 (non-identified), and *B. methylotrophicus* isolate 56 (Fig 3), may also be useful for the development of new products for the control of nematodes because similar behavior has been observed for some

commercial organophosphate and organo carbonate nematicides (Sikora et al., 2005). However, in longer exposition periods, such as 48 h and 72 h, almost all bacterial isolates from the *M. exigua* egg mass demonstrated very strong nematicidal effects against *M. exigua*. Therefore, the production of VOCs by the bacterial isolates of *M. exigua* egg masses reveals greater relevance than the fungal VOCs. Furthermore, only four and three hours of exposure of the *M. exigua* J2 to the VOCs produced by *Microbacterium* sp. and *Pseudomonas putida* isolated from *M. exigua* egg masses was enough to kill more than 50% of J2s, demonstrating that the second-stage juveniles are highly sensitive to the VOCs produced by both bacteria (Fig 5). However, there are no reports in the literature of the sensitivity of plant nematodes to the duration of the exposure times to VOCs.

The ability of fungi and bacteria of the *M. exigua* egg masses to produce VOCs reported in the present study demonstrates another mode of action of this microbiota in reducing the population of the coffee root-knot nematode *M. exigua*. In the field, the period of contact between eggs and J2s with VOCs is longer than the period studied here because the coffee crop is perennial. It should also be noted that VOCs may act at different stages of the egg embryonic development, especially at the stages in which the lipid barrier below the chitin layer does not exhibit high efficacy in preventing entry of toxic molecules inside the egg, such as in the final stages of the juvenile development and during the J1 and J2 stages still inside the egg (Perry, 2002). Costa et al. (2012), estimated 53% to 76% of eggs with abnormal juveniles and found 23 to 45% eggs with well-formed juveniles among those that did not hatch *M. exigua* J2s.

Therefore, *M. exigua* egg masses develop a microbial community that over time adapts to the particular niche, and the VOCs emitted by the resident community (fungi and bacteria) are toxic to the coffee nematode *M. exigua,* thus exerting biocontrol of these pathogen. In conclusion, the microbiota formed by fungi and bacteria occur in the *M. exigua* egg masses throughout the year, with a predominance of the fungi *F. oxysporum* and produce VOCs toxic to J2. The VOCs produced by the resident bacteria of *M. exigua* egg mass, *Pseudomonas putida* and *Microbacterium* sp. caused immobility and mortality in 50% of the hatched J2s after 3 and 4 hours of exposure, respectively, extending or interrupting the life cycle of the plant nematode *M. exigua.*

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ARTICLE 2 **Cucumber volatile emissions in above-below ground interactions among herbivores**: nematodes (*Sedentary endoparasites*), spider mites - and its natural enemies

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Artigo preparado de acordo com as normas do periódico *Plant Pathology.*

ABSTRACT

Although plants emit volatile organic compounds (VOCs) when they are affected by pathogen or herbivores either aboveground in the leaves or also belowground in the roots, the studies on the interaction among them have substantially increased. In this study we analyzed the profile of volatile compounds in above-belowground interactions between the nematode *Meloidogyne incognita* (N) and the two-spotted spider mite, *Tetranychus urticae* (S) in cucumber plants besides the attraction of the predatory mite *Phytoseiulus permisilis* (S-predator) by the emitted VOCs. Then second state juvenile (J2) of *M. incognita* produced in greenhouse was inoculated on cucumber plants. Twenty five days after J2 inoculation, three cucumber plants were infested with 20 spider mites (*Tetranychus urticae* reared in bean, *Phaseolus vulgaris*). Volatiles were collected separately from leaves and roots, thirty two days after J2 inoculation using dynamic headspace collector in greenhouse. Volatile induced assay on timing periods of J2 inoculation as well as trials with closed Y-tube olfactometer for testing attraction of S predator were underwent. *M. incognita* caused root galls in cucumber plants, but cucumber infested with spider mites (S) alone and combined with *M. incognita* J2 had significant reduction of total (root+shoot) and shoot biomasses changes in VOCs profile collected from cucumber roots were always higher than in the leaves evaluated by clusters analyses. In both organs, the altered compounds were mostly terpenes, alkanols, aldehydes and aromatic phenols either down regulated or up regulated. The overall molecules shifting was only detected by PCA analysis on roots infested by nematode

after 9 and 13 days after J2 inoculation. The most differences encountered were up-regulated in J2 inoculated cucumber roots compared to control. By olfactometer trials the attraction of the S predator (*P.persimilis*) by the S infested plant was strong regardless the presence or not of the nematode in the same plant. Nematode and spider mites infestations alter cucumber VOCs emittions and which are the cues for insect natural enemies attraction.

Keywords: induced plant volatiles, above-belowground interaction, nematodes, spider mites

Introduction

Plants are sedentary organisms during their life cycle and are constantly interacting with various others organisms including herbivorous, and pathogens on above-and belowground parts. The strategies of plants to survive such multiple attacks are due their diversified defense mechanisms, including structural and biochemical responses which reduce pathogen progress. Among them, are the morphological changes, production of defensive proteins or toxins, or release of volatiles that either have a repellent effect or attract predators of the attacking herbivores (Bezemer and van Dam, 2005; Heil, 2008; Dicke et al., 2009).

Insects and nematodes are so abundant and diverse, leading plants to interact with them via shared resources. The presence of these herbivore communities induces responses that influence plant quality which means systemic response and the subsequent preference and performance of the undesired organisms in different plant organs (Ohgushi, 2005; van Dam and Heil, 2011).

Physiological integration of roots and shoots in plant defense remained unrecognized for a long time (Kaplan et al., 2008a), especially on the defense mechanism involving the relationship between shoot insects and root nematodes. But in the last decade, both groups of herbivores (insects and nematodes) have been investigated as positive or negative, on the mechanisms of plant-mediated above-below ground interactions (Wardle et al., 2004; van Dam et al., 2005;De Deyn et al., 2007; Wurst and van der Putten, 2007; Kaplan et al., 2008a; Olson et al.,

2008; Lohmann et al., 2009; Hong et al., 2010; Vandegehuchte et al., 2010; Wondafrash et al., 2013). Interaction studies have been expanded to the mycorrhizal fungi (AMF), and belowground biota which resulted in a positive or negative plant response and thereafter influence herbivore performance and affect population dynamics as dispersal strategies (Hong et al., 2010; Bonte et al., 2010; Hong et al., 2011; De Roissart et al., 2013). These studies, along with others, suggested that the attacks by herbivore and/or pathogen can induce profound changes in the aboveground plant parts mainly in the distribution of metabololites within the plant and to the production of green leaf volatiles (Matsui, 2006; De Roissart et al., 2013).

Plants have been shown to emit volatile organic compounds (VOCs) when they are affected by pathogen or herbivores either aboveground, in the leaves or also belowground, in the roots (Jansen et al., 2011). Plant responses on interaction among diversified organisms (insects, nematodes, root pathogen and symbiotic organisms) between above-and belowground are known to be complex. Some resulting substances have been characterized as terpenoids, glucosinolates, or phenolics, both in the roots as well as in aboveground plant tissues (Manninen et al., 1998; Bezemer et al., 2004; Kaplan et al., 2009; van Dam, 2009). Application of volatile on the shoots promoted changes in alkaloids, phenols, amino acids and carbohydrates in the roots of coffee plants (Silva et al., 2013).

Releases of herbivore-induced plant volatiles (HIPV) provide new blends of different compounds that attract natural enemies, and many can discriminate between volatiles induced by different species of herbivores

(Dicke, 1999). Indeed, the composition of HIPV can be specific to the herbivore species, and moreover, many carnivorous arthropods can learn to discriminate between different volatile cocktail (Dicke et al., 1990a; De Boer and Dicke, 2004a; De Boer and Dicke, 2004b-b; De Boer et al., 2004; Kappers et al., 2005).Plants VOCs provide olfactory cues that attract carnivores enemies after infestation with herbivorous spider mites in cucumber varieties. The amount of (*E*)-ß-ocimene, (E,E)-TMTT correlated positively with the attraction of predatory mites (Kappers et al., 2011). However, little to nothing is known about the consequences of the changes on the plant volatile emissions in above-belowground interaction between nematodes and insect herbivores for the behavior and performance of the natural enemies. Therefore, in this study, we analyzed the profile of volatile compounds in above-belowground interaction between the nematode *Meloidogyne incognita* and the two-spotted spider mite, *Tetranychus urticae* in cucumber plants. In addition, we analyzed the attraction of the predatory mite *Phytoseiulus permisilis* to the volatiles emitted by cucumber plants infested by both *M. incognita* and *Tetranychus urticae*.

Material and Methods

Changes in volatile emission in above-belowground interactions.

Plant growth, Meloidogyne incognita and herbivores rearing(biomass)

Seeds of cucumber (*Cucumis sativus* accession ENZA1) were sown and seedlings were grown in a greenhouse, under a natural daylight photoperiod (December-January). Day and night temperatures were set at 23±2°C, with relative humidity (RH) of 60±10%. Four-wk-old plants with 3 to 4 leaves were used on the experiments. Ten days before nematode inoculation, 2 pipet tips were digged into the sand substrate (silver sand complemented with water and nutrients) at 1cm from the stem and 1.5cm depth. The hole formed, when the pipet tips were removed, was used to introduce the nematode inocula into cucumber rhizosphere for infestation.

Four-wk-old cucumber plants and *M. incognita* J2 were produced in the greenhouse. And the herbivore *Tetranychus urticae*, the two-spotted spider mite, was reared on bean plants (*Phaseolus vulgaris*) also in a greenhouse. Predatory mites, *Phytoseiulus persimilis*, were reared on spider-mite infested bean plants in a growth cabinet at 20 °C, and used for nematode and spider mite interactions studies.

Assay – J2 of *M. incognita* was inoculated in 3 cucumber plants as described. Twenty five days after inoculation, three J2 inoculated cucumber plants were infested with 20 spider mites equally distributed over the second and third leaves. Also healthy cucumber plants were infested by the same way with only spider mites. As a control, cucumber plants were not infested by either J2 nematodes or spider mites. Then we had four treatments. The time period to collect volatile was defined by thirty two days after J2 inoculation which means 7 days after spider mites infestation when the volatiles were collected, separately, from leaves and roots using dynamic headspace collector in the greenhouse. The quantity and composition of the volatiles were analyzed using thermo-desorption GCMS and expressed per gram fresh weight (FW). After collecting volatiles, root systems were harvested, washed gently and weighed. Galls were counted and the value was divided by the total root weight, giving galls per gram of roots. However, the main objective of this assay was to check, only, the nematode infectivity on cucumber plants. Biomass was also calculated by summing up root and shoot weights giving the total biomass. Aboveground biomass or belowground biomass was considered respectively leaves and roots FWs. The ratio was obtained dividing root FW by shoot FW. The assay was arranged in randomized block design with three replicates per treatment. Volatiles profiles in both shoots and roots analyzed by GCMS had the data pre-processed by peak picking and alignment (Metalign) following by statistical analysis using multivariate PCA (GeneMath) furnishing the total molecules shifting caused by nematodes or spider mite and both. In this context some metabolites were characterized through MSClust and NIST program.

Changes in volatile emissions after nematode-plant interaction (timing).

Plants and Nematodes - Seedlings of cucumber was produced as described and four-wk-old plants with 3 to 4 leaves were used on the experiments. The nematode *M. incognita* was used to induce volatile in cucumber plants. Ten days before nematode inoculation, 2 pipet tips were digged into the sand substrate (silver sand complemented with water and nutrients) at 1cm from the stem and 1,5cm depth. The hole formed when the pipet tips were removed was used to introduce the nematode inocula into cucumber rhizosphere.

Assay – The pipet tips placed close to the cucumber stem were removed and the hole was filled with 500 second stage juveniles (J2) in 1ml water suspension. One, 2, 3, 6, 9 and 13 days after J2 inoculation the quantity and composition of nematode-induced cucumber volatiles were analyzed in roots and shoots separately, using the dynamic headspace collector in the greenhouse. Volatiles were collected and analyzed at each time period in J2 inoculated cucumber and health cucumber plants (control). Thirty days after J2 inoculation, the root galls were observed in the root as a proof of nematode infection, once these data was not relevant for this assay. The assay was arranged in randomized block design with five replicates per treatment.

Predator Attraction Setup

Bioassay–Because the volatiles emitted by cucumber were altered by J2 infestation, the test was setup to evaluate the response of predatory mites to volatiles released by infested cucumber plants with J2 nematodes and/or spider mite compared to uninfested plants. These studies were underwent with a closed Y- tube olfactometer. Odor sources consisted of a single plant with 4-5 leaves infested with 15 spider mites seven days before the test or 500 J2 nematodes, 30 days before the test. Plants combined infested by the herbivores received J2 nematodes 23 days before spider mite infestation. As a control volatiles of uninfested (either by nematode or spider mites) were studied. Individual spider mite female predator was released on iron wire at the basal tube, and its behavior was observed for a maximum of 10 min. The connections of the odor sources to the arms of the olfactometer were interchanged after testing a series of five predators. A predatory choice was recorded when it reached the finish line, halfway up of the olfactometer arms, at a established time period. Otherwise it was recorded as no-choice. Each predator was used only once. Per experimental day, 20 predators were tested for each odor combination with plants at the same infestation time periods. Combinations were considered treatments. Every two combinations were done every day. In three days all combinations were studies per replicate. For the total experiment three replicates were used. Odor drived spider mites predator in the olfactometer Y-tube and thereafter combinations were selected for the assay. Then combinations were selected as following: 1) clean air was compared to uninfested and infested spider

mite (S) plants; 2) uninfested plants vs infested with (S) or J2 nematode (N); 3) infested plants (N) vs combined infested plants (N+S); 4) finally infested plants (S) with combined infested (N+S). The number of spider mite predator that reached halfway of the finish line was recorded. The x^2 probability test discriminated the combinations studied.

Volatile analysis – to analyze the quantity and composition of nematodes and herbivores-induced cucumber volatiles, dynamic headspace collector was used in the greenhouse setup. A glass jar connected to an inlet and outlet Tenax liner was placed over the plant. Volatiles were trapped for 1.5 hours and collected on Tenax® sorbent tubes combined with micro air samplers (PAS-500, Spectrex). Per time period, 5 plants were analyzed as well as a background sample. Tenax liners were analyzed on thermodesorption GC-MS.

Protocols:

Headspace analysis- Volatiles are trapped for 1.5 hours and collected on Tenax® sorbent tubes combined with micro air samplers (PAS-500, Spectrex). Volatile samples are analysed with a Thermo Trace GC Ultra connected to a Thermo Trace DSQ USA) quadruple mass spectrometer (Thermo Fisher Scientific, Waltham, MA) according to Kappers et al, 2011. In short: Tenax cartridges are dry-purged with nitrogen at 30 ml min^{-1} for 30 min at ambient temperature to remove water before desorption of the volatiles. Volatiles are desorbed from the Tenax cartridges using a thermal desorption system at 250°C for 3 min with a

helium flow of 30 ml min^{-1}. Volatiles are transferred in split mode (1:5) to the analytical column (Rtx-5 ms, 30 m, 0.25 mm i.d.,1.0 μm film thickness) by rapid heating of the cold trap to 250°C. The GC is held at an initial temperature of 40°C for 3.5 min followed by a linear thermal gradient of 10°C min^{-1} to 280°C, and held for 2.5 min with a column flow of 1 ml min^{-1}. The column effluent is ionized by electron impact ionization at 70 eV. Mass spectra are acquired by scanning from 45–400 m/z with a scan rate of 3 scans s−1. Compounds are identified by using the Xcalibur (Thermo Scientific) software in combination with the NIST 98 database libraries and by comparing their retention indices with those from the literature (Adams 1995; www.pherobase.com). For quantification, characteristic quantifier ions are selected for each compound. Metalign software (PRI-Rikilt, Wageningen, The Netherlands) is used to align peaks of chromatograms of all samples and to integrate peak areas for the quantifier ions.

Statistics- Individual volatiles were analyzed for significant changes among treatments using one-way ANOVA followed by a LSD *post-hoc* analysis (SPSS 15.0, Chicago, II, USA). The emitted quantities of individual volatile components were analyzed for significant differences among plant treatments by using a *t*-test(SPSS 15.0). Treatments were analyzed for significant differences using one-way ANOVA. Overall volatile profiles were analyzed by using Principal Component Analysis (GeneMath XT 2.0) after log_{10}-transformation of the whole dataset and subtracting the average value of all treatments to normalize the data (Hendriks et al., 2005). The first few principal components (PCs) explain

most of the variance in the original data, and we used it to visualize differences of the total volatile profile among treatments.

Multivariate mass spectra reconstruction (MMSR, Plant Research International, Wageningen, The Netherlands) as developed by Tikunov et al. (2005) was used to reduce the MetAlign dataset of 33.800 mass fragments. By using MMSR, clusters of related metabolite fragments that show similar patterns over different samples was recognized as so-called centrotypes on the basis of their overlapping patterns throughout the different samples, and the corresponding metabolites were subsequently identified.

Results

Changes in volatile emission in above-belowground interactions

Galls per gram of cucumber roots were not significant among treatments (i.e. J2 inoculated plants and simultaneously nematode and spider mites inoculated plants). The aboveground treatment (spider mites) had a significant effect on the plant biomass allocation and there were no significant differences in root biomass allocation (i.e. the root/shoot ratio) (Table 1). Nematode (N) treatments fail to decrease root biomass compared to control in all evaluations (total, shoot and root). However Spider m. (S) and combined infestation of both (Spider m. plus nematode-S+N) treatments reduced significantly total and shoot biomass compared to control and nematode treatment alone (Fig 1).

Table 1
Results of ANOVA of the measured plant biomass in relation to the belowground and aboveground treatment at 30 days after nematode and spider mites infestation.

Parameter	F	*P*
Total biomass (g)	29.12	< 0.0001
Aboveground biomass (g)	16.99	< 0.0001
Belowground biomass (g)	1.26	0.349
Root/shoot ratio	1.33	0.330

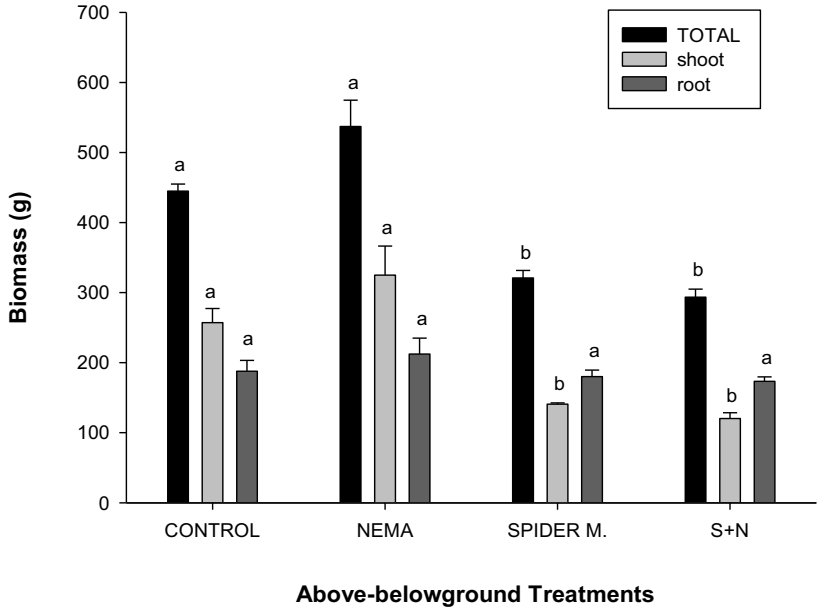

Fig 1 – Effects of the above-belowground biotic treatments on plant performance. Control: untreated plants, Nema: nematodes, S+N: Spider m. plus nematodes. Means followed by same letter (among treatments) are not significantly different according to TUKEY test at 5% probability.

Leaves and roots from cucumber plants emitted significantly higher or lower amounts of total volatile organic compounds (VOCs) compared to control plants after induction by different herbivores (nematode and/or spider mites). Although PCA did not demonstrate the class separation between S inducing VOCs and S+N inducing VOCs, they were separated from control and N inoculated plants alone (Fig 2).

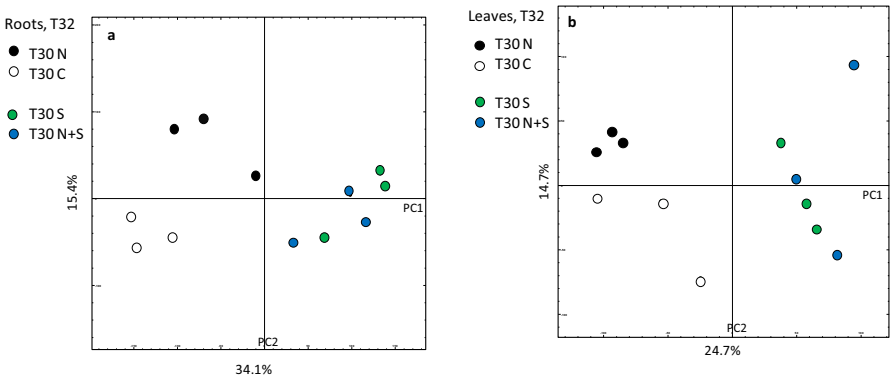

Fig 2 – a: Principal Component Analysis of the mass fragments of volatile emitted after nematode and/or spider mite infestation of leaves and roots material compared to non-infected plants (control). The first two principal components explains 24,7% of the variation found and the second PC 14,7%. **b**: root material: The first two principal components explains 34,1 of the variation found and the second PC 15,4. Treatment labels: "C", non-infested plant," N", nematodes, "S", spider mite, "N+S", nematode and spider mite.

However, the volatile profile of root material showed always higher changes in collected VOCs than leaf materials as observed by the cluster analyses (Fig 3). Most of the root released compounds were down-regulated for those most studied ones contrasting with leaf released compounds which were up-regulated. Altered compounds in leaves and roots included, among others, terpenes, alkanols, aldehydes, aromatic phenols, although all terpenes were not encountered in every plant organs (Table 2).

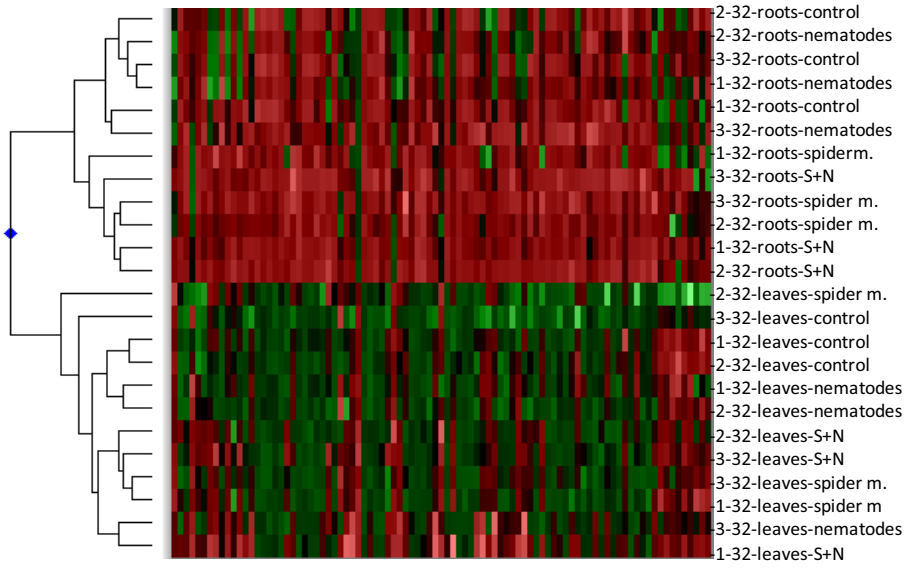

Fig 3 – A heat map for measuring volatile metabolites related to secondary metabolism. Differences between compounds level in control and above-belowground treatments were examined using Student's *t*-test, and only those that were significantly different at the $P< 0.01$ level were selected. Row represents treatments and column concentration of individual compounds. Red represents a high ratio and green a low ratio of individual compounds.

Table 2
Compounds potentially emitted by roots and leaves during infestation. Amounts emitted are denoted as GCMS area units indicating significant differences between non-infested plant and treatments.

			Leaf				Roots			
Species	**RI Exp.**	**RI Lit.**	**Nema**	**Spider M.**	**N+s**	**regulated activity**	**Nema**	**Spider M.**	**N+s**	**regulated activity**
Limonene	1032	1031,5	√			down	√			down
				√		up		√		down
					√	up			–	–
Linalool	1103	1098	–	–	–	–	√			down
			–	–	–	–		√		down
			–	–	–	–			–	–
Sabinene	975	974	–	–	–	–	√			down
			–	–	–	–		√		down
			–	–	–	–			√	down
ß-pinene	979	978	–	–	–	–	√			down
			–	–	–	–		√		down
			–	–	–	–			√	down
α-pinene	934	936,6	–	–	–	–	√			down
			–	–	–	–		√		down
			–	–	–	–			√	down
Camphene	950	951	–	–	–	–	√			down
			–	–	–	–		√		down
			–	–	–	–			√	down

Styrene	890	892	–	–	–	–	–	–	–	–
				√		up		√		up
					√	up			√	up
Acetophenone	1073	1065	–	–	–	–	–			–
			–	–	–	–		√		up
			–	–	–	–			√	up
1-Decene	1093	989,5	–	–	–	–	–	–	–	–
			–	–	–	–		√		up
			–	–	–	–			√	up
1-Hexanol	869	866,5	–	–	–	–	√			up
			–	–	–	–	–	–	–	–
					√	up	–	–	–	–
(E)-DMNT	1119	1114	–	–	–	–	–	–	–	–
				√		up	–	–	–	–
					√	up	–	–	–	–
Tetradecane	1402	-	–	–	–	–	√			down
			–	–	–	–		√		down
			–	–	–	–			√	down
Tridecanal	1516	1505,5	–	–	–	–	√			down
				√		down		√		down
			–	–	–	–		–	–	–

Changes in volatile emissions after nematode-plant interaction

The PCA analyses of volatiles from roots demonstrated initially significant volatile molecules shifting for both timing, 9 and 13 days from inoculation, and nematode infestation compared to control. These two variations: time (9 and 13 days) and nematode (inoculated plants and control) are, respectively, explained by 36,1% and 20,6% accounting for the total of 56,7% which is referred as good to discriminate the treatments. In leaves, on the other hand, volatile molecules shifting were not observed in J2 inoculated cucumber and healthy plants but only occurred on timing (Fig 5).

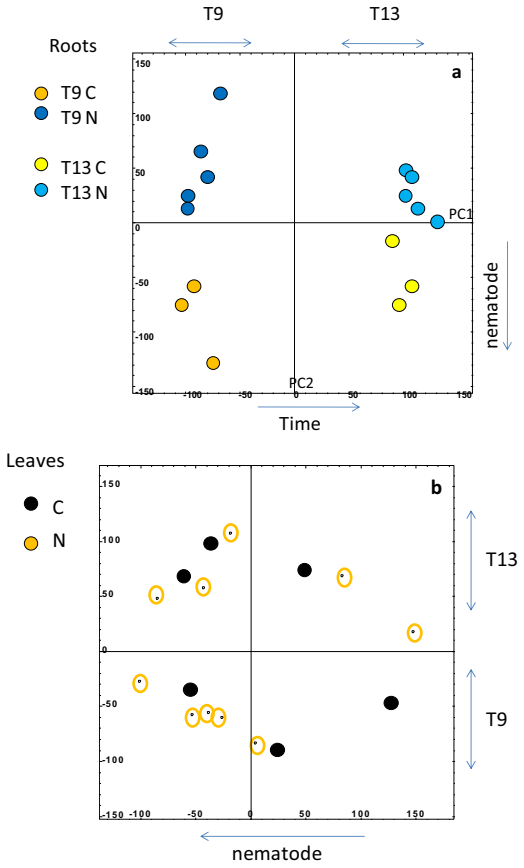

Fig 5 – a: Principal Component Analysis of the mass fragments of volatile emitted after nematode infestation of cucumber plants compared to non-infected plants. The first PC explains 36,1% of the variation found and the second PC 20,6%. **b**: leaf emitted volatiles did not differ significantly 9 and 13 days after inoculation. Treatment labels: "C", non-infested plant," N", nematodes.

Differences on volatile emittions on cucumber roots between healthy and inoculated cucumber plants were significant only after 9 days from inoculated regarding to some molecules as presented in the Figure 6. Depending on the molecule nature these significant differences occurred at 9 or 13 days from J2 inoculation. Most differences encountered were up-regulated in J2 inoculated cucumber compared to control. But in

contrast, acetophenone was down-regulated also compared to control (Fig. 6e). These differences were not studied for leaves, once the overall volatile molecule shifting was not observed as explained in Figure 5.

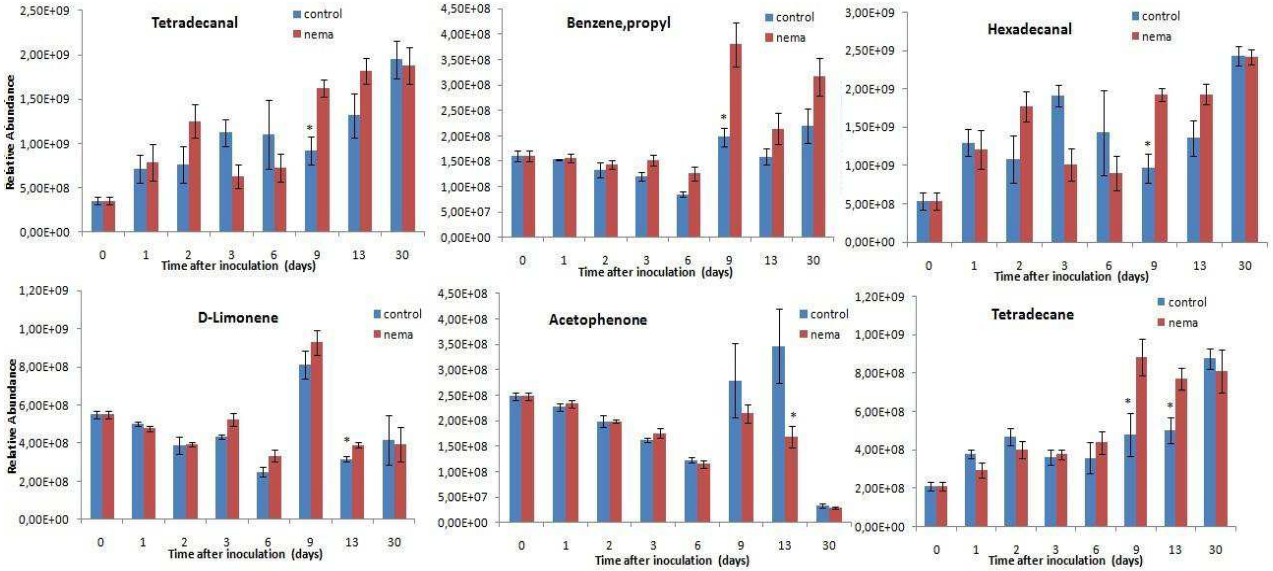

Fig 6 – Volatile emission of cucumber plants in response to nematode infestation in time. *Asterisks* indicate significant deviation from all other values (Tukey´s test, $P< 0.05$). Control untreated plants.

Olfactometer trials

The nematode infested plant fail to attract the natural enemy, S predator *Phytoseiulus persimilis,* (Fig 7d). The S predator was always attracted by the previously S infested plants alone or combined infestation with N (Fig 7b, c, e, f), although statistical significant attraction was only observed on S infested plants (Fig 7b). In short, of the S predator attraction by the S infested plant is strong regardless the presence or not of the nematode in the same plant.

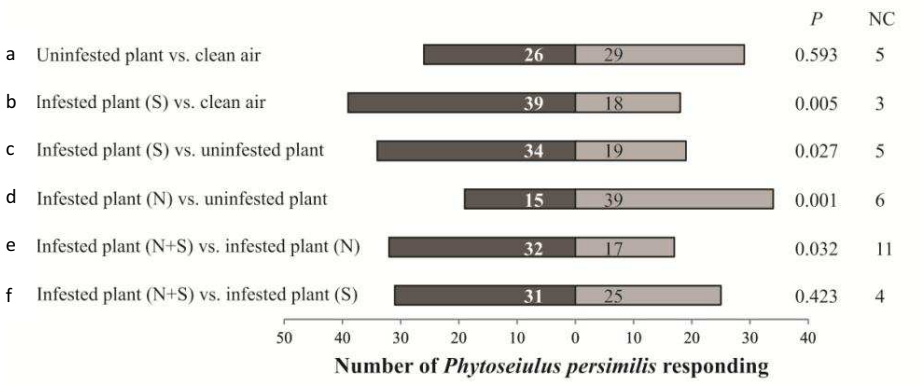

Fig 7 Mean number of decisions per individual of *Phytoseiulus permisilis* in the olfactometer experiments for plants after induction with nematodes and or spider mite. NC: no choice,indicates the number of tested individuals that did not make a choice. ns $P > 0.05$. Treatment labels: " N", nematodes, "S", spider mite, "N+S", nematode and spider mite.

Discussion

The significant reduction of total and shoot biomass by infestation with spider mite (S) and combined with J2 nematode (N), as well as the formation of root galls demonstrated great changes in plant physiology leading to disease in cucumber plants which are corroborated by researchers (Bonte et al., 2010; Vandegehuchte et al., 2010; De Roissart et al., 2013). Since they were infesting the same plant, interaction between them is might to occur. Cucumber herbivores (spider mite and nematode) with two different feeding modes and feeding locations on the shared host can interact indirectly via the host plant (Hong et al., 2010; Hong et al., 2011). In fact, the feeding strategy of the nematodes, the state of host plant susceptibility to herbivores and the sort of shoot herbivorous insects, are important factors determining the outcome of interactions between root-feeding nematodes and shoot herbivorous insects as these may differentially influence the responses of the host plants to attackers (Mateille, 1994; van Dam et al., 2003; van Dam et al., 2005; Bezemer and van Dam, 2005; Wurst and van der Putten, 2007).

The interaction mechanisms are always mediated through changes in host plant quality and/or the up-regulation of plant defense strategies (Masters and Brown, 1997; Van der Putten et al., 2001). Besides, the mechanisms of interaction between nematodes and spider mites are complex and may depend on variation in abiotic conditions at both temporal and spatial scales (Wardle et al., 2004; Vandegehuchte et al., 2010; Thamer et al., 2011).

The results from our experiments indicate that spider mites affected plant quality (Table 1) and the nematode caused cucumber root galls. In recent research, the results provide insights on the relationship between below-and aboveground herbivores on plants where biotic interactions at specific plant regions are expected to induce strong effects on plant quality, for example: root feeding by nematodes influences negatively decisions of an aboveground herbivore as oviposition, population dynamics and behavior performance or metabolic composition (Anderson et al., 2011; De Roissart et al., 2013). In our results the interaction between S and N caused distinct VOC classes in plants infested with S (alone or combined with N) as compared to infested plant with N alone and apparently, nematode infection had no effect on herbivore insect performance that means nematodes caused significant any changes in the metabolic composition of the shoots (Fig 2). Different species of organisms in the roots induce different class of volatiles that are released by the plant (Neveu et al., 2002; Rasmann et al., 2005; Soler et al., 2007; Ferry et al., 2007; Olson et al., 2008; Ali et al., 2011)or influence the concentration of plant defense compounds (Manninen et al., 1998; Bezemer et al., 2004; Kaplan et al., 2009; van Dam, 2009),although only a few studies on the role of volatiles induction by above-belowground feeding herbivores in the same plant have been done. For example Olson et al. (2008) found that in cotton plants root feeding by *M.incognita* has a little influence on direct and indirect defenses of *Gossypium hirsutum* against insect herbivory.

The higher changes of root volatiles profile along with decreased levels of infested cucumber with the herbivores studied in this work as

compared to leaves may reflect strong physiological changes in roots. Because roots are concealed belowground and spatially separated from leaves, their contribution to aboveground herbivore plant defense strategies is not generally acknowledged. Mechanisms underlying these interactions may be reallocation of primary plant metabolites such as nitrogen and carbohydrates to foliage in response to root feeders, or the induction of secondary metabolites (Bezemer et al., 2004; Kaplan et al., 2008a). However, nematode infection may have either beneficial, neutral or detrimental effects on herbivore performance, and likewise herbivory may affect pathogen growth (Ponzio et al., 2013). Root nematode herbivor is known to suppress the constitutive and inducible foliar expression of nicotine in tobacco (Kaplan et al., 2008b), and therefore some works suggested that root-fedding herbivores would induce susceptibility to leaf-feeding insects by interfering with aboveground compounds dynamics (Kaplan et al., 2008a).It is clear that the outcome of such interactions is dependent on the plant, herbivore and feeding strategy involved (Ponzio et al., 2013).

Some of the volatiles in leaves and roots are higher or low levels in our assay (Table 2). Kutyniok and Muller (2012), found that in aphid treated plants, several metabolites including glucosinolates, important defence compounds of Brassicaceae, were reduced in the shoot, but only minute changes took part in the systemic root tissue, and nematodes did not cause significant changes in the metabolic alteration locally or systemically. In contrast, Crespo et al. (2012)studied detection of root-induced volatiles in *Brassica nigra* plants infested with *Delia radicum* L. root fly larvae and found methanethiol, dimethyl sulfide (DMS) and

dimethyl disulfide (DMDS) levels increased much later in infested roots, indicating that activation of enzymes or genes involved in the production of these compounds may be required. Altered volatile compounds found in the leaves of cucumber infested by herbivores such as terpenes: linalool, DMNT and limonene were also found by Arimura et al. (2005), Dicke et al. (2009), Kappers et al. (2010), Kappers et al. (2011). Some authors have suggested that microorganism's infestation may enhance specific biosynthetic pathways, for example the methyl-D-erythriol 4-phosphate pathway (MEP pathway) that leads to the monoterpenes cis-β-ocimene and linalool (Mithofer et al., 2004; Dudareva et al., 2005; Hampel et al., 2005; Mithofer et al., 2005) or the mevalonate pathway (MVA pathway) producing sesquiterpenes such as (E)-β-caryophyllene, β-bisabolene e DMNT (Kessler, 2011; Paetzold et al., 2010). In our work, we showed that herbivores infestation may enhance specific biosynthetic pathway and some compounds were little or negatively affected (Table 2).

The occurrence of molecule shifting in the roots after 9 and 13 days, only from inoculation of the N (Fig 5a) may demonstrate the time elapsed from the herbivore feeding initiation to fulfill the host changes. The greater difference on volatile emission from cucumber roots only after 9 days from the inoculation (Fig 6) may be related to the time required by the plant to respond to the damage caused by the N infestation. Some authors suggest that in leaves infested by the early HIPV are usually the fatty acid-derived GLV (green-leaf volatiles) such as C6 aldehydes, C6 alcohols and C6 esters, whereas terpenoids usually are emitted later (Dicke et al., 2009). In the literature, the most emitted

VOCs came from the whole plants. One of the first study with detection of root-induced volatiles in *Brassica nigra* suggested that induction process involving the transcription of genes or activation of enzymes require time periods after infestation (Crespo et al., 2012). Thus, the difference between the sorts of compounds found on the cucumber roots could be related to the time of root volatile emission after pathogen induction.

Some specific molecule had a lower level in our assay, such as acetophenone (Fig 6e). The volatiles chemicals produced by plants include a wide variety of short-chain alcohols, aldehydes, ketones, esters, aromatic phenols, and lactones, as well as mono- and sesquiterpenes (Simpson, 1994).Some of them have been proved to be toxic to nematode. For example different isomers of alkanols are toxic to *Bursaphelenchus xylophilus* (Seo et al., 2010).

Changes in plant chemistry in response to herbivory are generally mediated by phytohormone signalling (Pieterse and Dicke, 2007).By knowing that there is potential for crosstalk between the salicylic acid (SA) and jasmonic acid (JA) signalling pathways in particular, it is likely that interactions between attackers of different feeding strategy can also affect induction of indirect plant defences. It is thought that crosstalk between signalling pathways are essential for the fine-tuning of plant responses to specific attackers.

In the roots, *M. incognita* infection of *A. thaliana* strongly induces the SA-controlled defense at day 9 and 14, but not at day 5. In addition, a weak response of JA- controlled defense markers was observed at day 9 (Hamamouch et al., 2011). Sedentary endoparasitic nematodes seem to

initially induce the JA, ET, an SA pathways, but very quickly, especially after *M. incognit*a infection, parts of these pathways are repressed again (Wondafrash et al., 2013). It is likely that the results about the interaction between nematodes and spider mites have been regulated for the same JA signalling pathway since did not alter the volatile blend induced by spider mites from the leaves, suggesting an absence of negative crosstalk in this system. Dual induction of the JA signal-transduction pathway may explain the enhanced VOC emissions. Clearer answers to these interactions could be found by measuring the phytohormone concentration in response to spider mite or nematode feeding. The infection of peanut plants by necrotrophic pathogen Sclerotium rolfsii, thought to primarily induce JA, had no negative effect on VOC emissions in response to herbivory by *Spodoptera exigua* caterpillars. Along with pathogen-induced volatiles, the plants emitted all the compounds that are produced in response *to S. exigua* herbivory alone, often in greater amounts (Cardoza et al., 2002). Later (Cardoza et al., 2003a) showed for this system that dually challenged plants had significantly higher levels of JA than the expected additive effect of the individual attackers.

The strongest attraction of *Phytoseiulus persimilis* by the S infested cucumber regardless the nematode presence in the same plant contrasting to non attraction by the N infested plant (Fig 7) demonstrated the evolution of its sense of smell as predator toward the food source (spider mites). Attraction of predator to the prey is done always by the emitted plant volatiles (Vet and Dicke, 1992; Dicke, 1999; Heil, 2008; Dicke and Baldwin, 2010). For example Zhang et al. (2009) studied the behavior of predatory mites and showed a strongly diminished response

to plants infested by spider mites and nonprey whiteflies. Dually damaged plants were shown to have reduced levels of (E)-β-ocimene emission, which is a compound known to be attractive to the predator (Dicke et al., 1990a). Absence of a key compound can lead to a greatly diminished response of a predator (Ponzio et al., 2013). The reduction in (E)-β-ocimene emission in response to whitefly infestation correlated with reduced JA titre and reduced transcription of the JA-regulated gene encoding for the enzyme ocimene synthase that is crucial for in (E)-β-ocimene production (Zhang et al., 2009).Indeed, the volatile analyses indicate that spider mite feeding on cotton leaf tissue induces release of compounds known to be attractants for *P. persimilis,* and that adding nematodes do not change the level of these emissions. The attractiveness of typical spider mite-induced plant volatiles has been reported for (E)-β-ocimene, methyl salicylate, linalool, nerolidol, (E)-4,8-dimethyl-1,3,7-nonatriene [(E)-DMNT], and (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene [(E,E)-TMTT] (Dicke et al., 1990a; De Boer and Dicke, 2004a; De Boer and Dicke, 2004b-a; De Boer et al., 2004; Kappers et al., 2005; Kappers et al., 2011).Moreover, van Wijk et al. (2008)suggest that *P. persimilis* does not have an innate preference for (individual) HIPV, but is able to adjust its olfactory response based on experience. The presence of a specific compound as (E)-DMNT could explain our results evidencing that their emissions may have some consequences for parasitoid attraction and the dual attackers (nematode plus spider mite) just prove that this interaction may be able of regulate the same JA signalling pathway, contributing synergistically to the attractiveness process.

Nematodes and spider mites infestation in cucumber changes the overall physiological pathways of the plant, altering specifically the cucumber VOCs emittions. These volatiles induced are the cues for insect natural enemy attraction.

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