Plant Volatiles Reduce the Viability of the Root-Knot Nematode *Meloidogyne incognita* Either Directly or When Retained in Water

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Abstract

Volatile organic compounds (VOC) produced by green residues for the management of plant-parasitic nematodes are poorly studied for oilseed plants and some *Brassica* spp. To investigate the activity of VOC in vitro and as biofumigants, dry and aqueous macerates of broccoli (*Brassica oleracea* var. *italica*) shoots and sunflower (*Helianthus annuus*) seed were used against the root-knot nematode *Meloidogyne incognita*. VOC produced by sunflower seed caused higher mortality of *M. incognita* second-stage juveniles (J₂) than VOC produced by broccoli shoots but both plant species were equally effective in decreasing the infectivity and reproduction of this nematode. The number of galls and eggs produced by the nematode in tomato roots was reduced by 89 and 95%, respectively, on average, at the highest concentrations of broccoli and sunflower seed macerates tested as biofumigants. When nematodes were placed in water exposed to broccoli VOC, J₂ immobility increased and the number of galls and eggs produced by the nematode in tomato roots

decreased 80 and 96%, respectively. Water exposed to sunflower seed VOC had no effect on the viability of the nematode. Gas chromatography was used to identify five and six chemical groups in broccoli and in sunflower seed macerates, respectively, but only alcohols, sulfated VOC, and terpenes were detected in the water exposed to these plant macerates. Sulfated VOC from the water exposed to broccoli macerates were found to be involved in its activity against *M. incognita*. The purified VOC dimethyl disulfide (DMDS) and 3-pentanol were tested directly against J₂ and showed a lethal concentration of 176 and 918 µg/ml (ppm), respectively, whereas dimethyl sulfide had no effect against *M. incognita*. Furthermore, DMDS and 3-pentanol retained in water killed J₂ and reduced gall formation and the number of eggs of *M. incognita* on tomato roots. Both these plant species produced toxic VOC to *M. incognita*, whereas only VOC retained in water exposed to broccoli had activity against *M. incognita*.

The root-knot nematode *Meloidogyne incognita* causes economic damage to a wide range of economically important crops and is considered one of the most significant plant pathogens in agriculture worldwide (Abad et al. 2008; Jones et al. 2013; Trudgill and Blok 2001). The control of this plant-parasitic nematode has been achieved by the application of chemical nematicides when plant resistance is not available. There is a great interest in using environmentally friendly strategies to control plant-parasitic nematodes such as plant amendments, green fertilization, or biofumigation (Caboni and Ntalli 2014; Oka 2010; Ploeg 2000).

Animal and plant residues have been applied as soil amendments to control a wide range of soilborne pathogens, including nematodes (Bailey and Lazarovits 2003). One such strategy is biofumigation. In this method, plant residues are incorporated into the soil and the chemical compounds with biocidal activity released during tissue degradation control pathogens. Some of these toxic chemicals are volatile organic compounds (VOC), which are gases or liquids that may evaporate under normal conditions of temperature and pressure. VOC are characterized by having carbon in their composition, low boiling points, and a lipophilic nature (Baldwin et al. 2006; Heil and Ton 2008; Loreto et al. 2014). The production of VOC is influenced by environmental conditions, availability and source of nutrients, oxygen, moisture, and the microbial community (Dudareva

et al. 2013; Insam and Seewald 2010; Leff and Fierer 2008). Soil biofumigation is often done with residues of *Brassica* spp. because these plants contain glucosinolates that are converted into toxic compounds such as isothiocyanates (Ploeg 2008; Ploeg and Stapleton 2001). Examples include the use of mustard meal to control *M. chitwoodi* in potato and forage turnip (*Brassica rapa* var. *rapa*) and canola (*B. napus*) to control soilborne pathogens, including nematodes (Al-Rehiyani et al. 1999; Henderson et al. 2009; Potgieter et al. 2013). Thousands of VOC produced by plants are already known, representing around 1% of their secondary metabolites (Dudareva et al. 2006; Loreto et al. 2014). Different plant parts produce VOC with distinct ecological roles such as deterrents of herbivores (Dicke and Loon 2000) or with toxicity to plant-parasitic nematodes (Gu et al. 2007; Huang et al. 2010; Jardim et al. 2018; Zou et al. 2007).

Residues and the essential oil fractions of oil-producing species (also known as oleaginous plants) have shown activity against plant-parasitic nematodes (Oka et al. 2000a). Oilseed cakes such as sunflower (*Helianthus annuus*) cakes may be used to control plant-parasitic nematodes and fungi (Ganaie et al. 2011; Giudici et al. 2000; Tiyagi and Ajaz 2004). However, the VOC involved in the inhibition of pathogens were not identified.

Previous investigations have shown that some VOC produced by plants and microorganisms may be retained in the water, resulting in a toxic solution against plant-parasitic nematodes (Barros et al. 2014b; Grimme et al. 2007; Silva et al. 2017; Terra et al. 2017). Nematodes need water to move through the soil. Toxic VOC once retained in water may act as a liquid nematicide. However, few studies in vivo were done to investigate the water exposed to VOC (Terra et al. 2018).

In this study, our goals were to determine the effects of VOC from broccoli shoot and sunflower seed macerates against *M. incognita*. Nematodes were directly exposed to the VOC or to the water retaining VOC produced by these plant species. Furthermore, broccoli and sunflower seed were tested as biofumigants against *M. incognita* to verify the effect of VOC in combination with non-VOC. The volatility of broccoli and sunflower seed macerates and the VOC retained in water were determined by gas chromatography (GC) and some

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purified compounds were tested both directly and in water solution against *M. incognita*.

Materials and Methods

Plant material and preparation of macerates. Broccoli (*B. oleracea* var. *italica* 'Avenger') shoots (leaves, inflorescences, and stems) and sunflower (*Helianthus annuus* 'Catissol') seed were collected from the horticulture and oleaginous plants sector of the Agriculture Department, Federal University of Lavras, Lavras, MG, Brazil. Sunflower seed were collected when their moisture was 18% and broccoli shoots from 90-day-old field-grown plants. Seed and shoots were stored for a maximum of 24 h at 7°C before processing. Then, 15 g of sunflower seed or broccoli shoots were weighed, surface sterilized by immersion in 2% NaOH for 1 min, and washed with distilled water. Each sample was immersed in 100 ml of distilled water for 24 h, then macerated with a kitchen blender for 20 s and filtered through gauze (Barros et al. 2014b). The resulting liquid was considered the aqueous macerate (15 g of shoots or seed in 100 ml of water). For the dry macerate, 15 g of shoots or seed were surface sterilized as described above, blotted dry, and ground with a mortar and pestle. This ground material was considered the dry macerate. Dry and aqueous broccoli shoot macerates and sunflower seed macerates were used right after preparation to obtain the VOC in subsequent experiments.

Nematode inoculum. *M. incognita* race 3 was used in all experiments. Pure populations of *M. incognita* race 3 were maintained on tomato plants (*Solanum lycopersicum* 'Santa Clara') in a greenhouse. The species was previously identified by esterase patterns (Carneiro et al. 2005). Eggs were obtained according to Hussey and Barker (1973). These eggs were incubated in hatching chambers at 28°C and only 24-h-old second-stage juveniles (J_2) were used in the experiments.

Mortality and infectivity of *M. incognita* exposed to plant VOC. These experiments were conducted according to a previously described procedure (Barros et al. 2014b). Supelco vials (40-ml vial, 29 mm in diameter by 82 mm high, with silicon screw cap; Sigma-Aldrich, St. Louis) were half filled with 25 g of sterilized sand and 2 ml of distilled water. A 1.5-ml microcentrifuge tube (Eppendorf Biotech, Hamburg, Germany) was introduced midway into the sand. Aliquots of 2 ml of aqueous or 1.5 g of dry macerates of either broccoli or sunflower were placed on the sand surface around the microcentrifuge tube (Supplementary Fig. S1). The vials were sealed and incubated at 25°C in the dark for 3 days to allow the emission and accumulation of VOC. Then, 1 ml of a suspension containing 100 J_2 of *M. incognita* was placed inside the microcentrifuge tube with a syringe and nematodes were exposed to the VOC at 25°C for 3, 6, 12, 24, 48, or 72 h. Each sampling time consisted of an independent tube that was destructively sampled. Controls consisted of 2 ml of distilled water applied to the sand surface at each period. After each exposure period, vials were opened and the whole J_2 suspension contained in the microcentrifuge tubes was transferred to enzyme-linked immunosorbent assay (ELISA) microplates (96 wells) to determine the percentage of mobile and immobile nematodes under an inverted light microscope at $\times 20$ magnification. Nematodes that remained immobile 24 h after removal from the microcentrifuge tubes were considered dead.

To evaluate the infectivity of *M. incognita*, 1 ml of a suspension containing 500 J_2 was exposed to VOC emitted by dry and aqueous sunflower seed macerates, as described above. Only sunflower seed were used because this species had a stronger effect on J_2 mortality than broccoli. Controls consisted of 2 ml of distilled sterilized water instead of plant macerate. After the exposure to VOC for 3, 6, 12, 24, 48, or 72 h, the suspensions were collected, adjusted to 4 ml with distilled water, and equally divided with a pipette into four holes around the stems of 20-day-old susceptible Santa Clara tomato seedlings planted in Styrofoam trays with 72 cells (75 cm³) filled with Multiplant substrate (60% pine bark, 15% vermiculite, and 25% humus; Terrado Paraíso, Holambra, SP, Brazil). The water content of the substrate was 60% of the field capacity (± 15 ml/cell). The inoculated seedlings were transferred to a greenhouse and received irrigation

and fertilization according to the technical recommendations (Terra et al. 2017). The number of galls and eggs per gram on the roots was evaluated 45 days after the inoculation. Eggs were extracted from roots according to Hussey and Barker (1973), followed by the centrifugal-flotation method (Jenkins 1964), and counted in a Peters chamber under an inverted light microscope at $\times 20$ magnification. Egg densities were expressed as number of eggs per gram of fresh root.

The mortality experiments included three types of macerates (dry, aqueous, and control), two plants (broccoli and sunflower), and six periods of time, totaling 36 treatments. The experiment to evaluate infectivity was done with sunflower seed macerates (dry, aqueous, and control) and six periods of time, each one replicated six times, totaling 18 treatments.

Greenhouse biofumigation experiments. Plastic cups (300 ml) were filled with a mixture of different amounts of Multiplant substrate and 0, 1.2, 2.4, 4.8, or 9.6 g of dry broccoli or sunflower seed macerate, which represented 0, 1, 2, 4, and 8% of the total volume of each cup. The water content of the soil was set at 60% of the field capacity in each cup (± 40 ml). After that, 5 ml of a suspension containing 5,000 *M. incognita* eggs was added and the contents were mixed.

To test the effect of the VOC emitted by the macerates incorporated in the substrate on *M. incognita* J_2 , one microcentrifuge tube was buried halfway to the surface of the mixture and the cup was covered with polyethylene PVC film (0.009 mm thick; PVC Alfilm Protect, Alpes Ind., Goias, Brazil) to prevent the escape of VOC. Three days later, 1 ml of a suspension containing 100 *M. incognita* J_2 was injected inside each microcentrifuge tube through the PVC film with a syringe. The perforation was sealed with adhesive tape to retain the VOC and, 48 h later, the contents of the microcentrifuge tubes were transferred to ELISA microplates to evaluate the number of mobile, immobile, and dead J_2 , as described above.

After removing the PVC film and the microcentrifuge tubes, a 30-day-old susceptible Santa Clara tomato seedling was transplanted to each cup containing the mixture of macerates and the *M. incognita* eggs. Seedlings were kept in a greenhouse for 45 days before evaluation of the number of galls and eggs per gram of root, as described above. The experiments consisted of broccoli and sunflower seed macerates at five concentrations each, totaling 10 treatments, and were replicated six times.

Effect of water exposed to VOC on *M. incognita*. Three experiments were conducted to study the effects of exposed water to broccoli VOC. In one experiment, sterile distilled water was exposed to VOC produced by dry broccoli and sunflower seed macerates in polystyrene Petri plates (90 by 15 mm; Kasvi, São José do Pinhais, PR, Brazil) with two compartments (Barros et al. 2014b; Fernando et al. 2005). Dry broccoli or sunflower seed macerate (5 g) was placed in one compartment and water (1 ml) was placed in the other compartment. Plates were closed, sealed with PVC film, and incubated at 25°C for 3 days. Controls were distilled sterile water rather than plant macerates. The water exposed to the VOC and the control were removed from the plates and placed in 2-ml microcentrifuge tubes along with 0.5 ml of a suspension containing 100 *M. incognita* J_2 . The microcentrifuge tubes were closed and incubated at 25°C for 48 h and the numbers of motile, immobile, and dead J_2 were counted. The experiment had three treatments (broccoli, sunflower, and control) and treatments were replicated six times.

In a second experiment, J_2 were exposed for different durations to the water containing VOC produced by dry broccoli shoot macerate in polystyrene plates. For this, 5 g of dry broccoli shoot macerate was placed in one compartment of the plate and 1 ml of sterile distilled water was placed in the other compartment. The plates were sealed with plastic film and incubated at 25°C for 6, 12, 24, 48, and 72 h. Controls were plates containing only water in one compartment for the same periods of exposure. At the end of each period, the water was transferred to 2-ml microcentrifuge tubes along with a suspension of 0.5 ml containing 100 *M. incognita* J_2 . The tubes were closed and incubated at 25°C for 48 h, after which the number of mobile, immobile, and dead J_2 was counted. Sunflower-exposed water was

excluded from the subsequent experiments because it was not toxic to *M. incognita*. The experiment had two treatments (water and broccoli) with six exposure times per treatment and was replicated six times.

The infectivity and reproduction of *J*₂ in water exposed to broccoli VOC for 1 and 3 days were studied as described by Terra et al. (2017) in a third experiment. Thirty-day-old Santa Clara tomato seedlings grown in Styrofoam trays with 72 cells (75 cm³) filled with Multi-plant substrate were inoculated with 300 *J*₂ in a 2-ml suspension. The *J*₂ were equally distributed in four 1-ml pipette tips (0.5 ml/tip) halfway buried into the substrate around the stems of the seedlings. Subsequently, 4 ml of water exposed to VOC was applied to these pipette tips (1 ml/tip) and, 24 h later, an additional 4 ml of water exposed to VOC was added to the same pipette tips. The same volume of distilled water was used as the control treatment. Plants were kept in the greenhouse and the number of galls and eggs per gram of roots was quantified 40 days after the inoculation. Eggs were extracted according to Hussey and Barker (1973) and counted with a light microscope in a Peter's chamber. This experiment had three treatments (1 and 3 days of exposure and control) and was replicated six times.

Volatilome analyses by solid-phase microextraction GC mass spectrometry. The extraction of VOC was performed by placing

1.5 g of dry or 5 ml of aqueous macerate of broccoli shoots or sunflower seed in Supelco vials. Additionally, 5 ml of water exposed for 72 h to VOC emitted by broccoli shoot macerate and sunflower seed macerate was also used for VOC extraction in 20-ml Supelco vials. The extraction of VOC from the samples in the Supelco vials (dry and aqueous macerates and the exposed water of each plant species) was performed using Headspace solid-phase microextraction (SPME) (Arthur and Pawliszyn 1990) with the following parameters: fiber divinylbenzene, carboxen, and polydimethylsiloxane; extraction temperature of 55°C and sample agitation at 250 rpm; and extraction time of 35 min and desorption time in the GC injector of 2 min. For separation and identification of VOC, a gas chromatograph coupled to a GC mass spectrometry QP 2010 Ultra mass spectrometer (Shimadzu, Kyoto, Japan), equipped with an AOC-5000 automated injector for liquids and gases (Shimadzu) and an HP-5 column (30 m by 0.25 mm by 0.25 µm, 5% phenyl and 95% dimethylsiloxane), was used. Temperatures were 250°C for the injector, 240°C for the interphase, and 200°C for the ion source of the detector. The injector was operated in splitless mode or in split 1:4 mode, according to the intensity of the peaks in the samples. Helium grade 5.0 at 1 ml/min was used as a carrier gas. The temperature programming in the GC oven was set from 40 to 160°C at 3°C/min and then to 240°C at 10°C/min. The mass spectrometer was operated in scanning

Fig. 1. Effects of volatile organic compounds (VOC) from plant macerates on *Meloidogyne incognita* in relation to the time of exposure. **A**, In vitro evaluation of VOC from dry and aqueous macerates from sunflower seed in the mortality of *M. incognita* second-stage juveniles (*J*₂). **B**, In vitro evaluation of VOC from dry and aqueous macerates from broccoli shoots in the mortality of *M. incognita* *J*₂. **C**, Number of galls and **D**, number of eggs in tomato roots produced by *J*₂ exposed to VOC from dry and aqueous macerates of sunflower seed. Number of galls and eggs determined 45 days after the inoculation of 500 *J*₂ on tomato roots (*Solanum lycopersicum* 'Santa Clara'). Controls were the *J*₂ exposed to distilled water. Error bars represent the standard error of the means.

mode in the range of 40 to 350 atomic mass units. To identify VOC in the plant samples, the mass spectra of each peak of the chromatogram were extracted through the software Automated Mass Spectral Deconvolution and Identification System, v. 2.63. The identification of VOC was performed by comparing the mass spectra of the sample peaks with spectra from the National Institute of Standards and Technology (NIST) library using the software Mass Spectral Search Program (v. 1.7; NIST, Washington, DC) and by comparing the retention indexes obtained experimentally with the retention indexes found in the literature (Adams 2007; NIST 2016). Only peaks with a similarity higher than 80% between mass spectra were considered in comparisons. The experimental retention indexes were obtained through injection of a homologous series of alkanes.

Selection and activity of purified VOC. Three VOC were selected among the ones detected in the water exposed to broccoli macerates. These VOC were chosen because only water exposed to VOC from broccoli showed activity against *M. incognita*. Only VOC detected in both dry and aqueous macerates and in the water exposed to the dry macerate were chosen. Purified dimethyl sulfide (DMS; $(\text{CH}_3)_2\text{S}$, anhydrous, $\geq 99.0\%$), dimethyl disulfide (DMDS; CH_3SSCH_3 , anhydrous, $\geq 99.0\%$), and 3-pentanol ($(\text{C}_2\text{H}_5)_2\text{CHOH}$, anhydrous, $\geq 99.0\%$) were acquired (Sigma-Aldrich) and used in the in vitro and in vivo experiments described below.

This experiment was done in 1.5-ml microcentrifuge tubes containing 0.5 ml of each VOC diluted in water at different concentrations and 0.5 ml of water with 100 *J*₂. DMS and 3-pentanol were diluted in water containing 0.01% Tween 20 at final concentrations

of 600, 800, 900, 1,000, and 1,100 ppm ($\mu\text{l/ml}$), whereas DMDS was diluted at 200, 300, 400, 500, and 700 ppm ($\mu\text{l/ml}$). These concentrations were determined on the basis of pilot tests (data not shown). Controls consisted of water with 0.01% Tween 20 without VOC (0 ppm). The tubes were closed and incubated at 25°C for 48 h and, after that, the *J*₂ were collected on a 500-mesh sieve, washed with distilled water, and transferred to ELISA plates, and the number of mobile, immobile, and dead *J*₂ was determined as described above. The experiment had six treatments and treatments were replicated six times.

In another experiment, distilled sterile water was exposed to the purified VOC. Polystyrene Petri plates (90 by 15 mm; Kasvi) divided into two compartments were used in this experiment. In one compartment, 150 μl of each purified VOC was applied on the surface of a sterile cellulose filter paper (1:11 μm porosity) and 1 ml of water was placed in the other compartment. To test the mixture of the three VOC, 50 μl of each was applied on three separate pieces of filter paper in the same compartment. The plates were sealed with plastic film and incubated at 25°C for 72 h. Controls were plates containing only water in one compartment for the same exposure period. After incubation, the water was transferred to 2-ml microcentrifuge tubes along with a 0.5-ml suspension containing 600 *M. incognita* *J*₂. The tubes were sealed and incubated at 25°C for 48 h, after which the number of mobile and immobile *J*₂ was evaluated in 250 μl of the suspension (approximately 100 *J*₂). The remaining suspension (approximately 500 *J*₂) was inoculated around the roots of 20-day-old tomato seedlings. The seedlings were kept in a greenhouse for 40 days after the

Fig. 2. Soil biofumigation with sunflower seed and broccoli shoots at different concentrations (0, 1, 2, 4, and 8%) in the substrate. **A**, Immobility and **B**, mortality of *Meloidogyne incognita* second-stage juveniles (*J*₂) after exposure to volatile organic compounds from plant macerates. **C**, Number of galls and **D**, number of eggs in tomato roots cultivated in soil infested with 500 *J*₂ of *M. incognita* under biofumigation. After the biofumigation, a tomato seedling (*Solanum lycopersicum* 'Santa Clara') was transplanted to each cup and they were cultivated in the greenhouse for 45 days before the evaluations. Bars represent the standard error of the means.

inoculation and the number of galls and eggs per gram of root was determined as described above.

Data analysis. All experiments were performed twice. No differences were observed between repeats for all experiments by analysis of variance interaction (experiment 1–experiment 2). Therefore, a combined analysis was performed for every two experiments, and the data presented are the combined results of the repeats. Then, results from all experiments were submitted to the normality test of Shapiro-Wilk and to the test of variance homogeneity of Bartlett. Analyses of variance with the *F* test were performed after assuring that the data fit the normal distribution. For data sets with a significant *F* test ($P < 0.05$), the mean values of different treatments were compared with the Tukey test ($P < 0.05$). The analyses were done by the statistical and graphic program Sigma-Plot, version 12.0.

Quantitative treatments such as different periods of exposure to VOC and different concentrations of plant macerates or VOC were evaluated by linear or nonlinear regression and the curves were chosen by their best fit by the program SigmaPlot, version 12.0. Lethal concentrations (LC₅₀) of purified compounds were calculated by Probit Analysis by the program Excel.

Results

Mortality and infectivity of *M. incognita* exposed to plant VOC. VOC produced by both dry and aqueous sunflower seed caused higher mortality of *M. incognita* J₂ than VOC produced by broccoli shoot macerates ($P < 0.01$) (Fig. 1). The increase in the periods of exposure to VOC emitted by sunflower seed from both dry ($P < 0.01$) and aqueous macerates ($P < 0.01$) significantly increased

Fig. 3. Toxicity of water exposed to volatile organic compounds (VOC) from plant macerates. **A**, Immobility and mortality of *Meloidogyne incognita* second-stage juveniles (J₂) treated with water exposed to VOC from broccoli shoots and sunflower seed. **B**, Effect of different times of exposure of water to VOC from broccoli shoots on *M. incognita* J₂. **C**, Number of galls and of eggs in tomato roots inoculated with 500 J₂ treated with water exposed to broccoli VOC for 1 or 3 days. Evaluations of galls and eggs were performed 45 days after the inoculation of 500 J₂ on tomato roots (*Solanum lycopersicum* 'Santa Clara'). Mean values followed by the same letter are not significantly different according to Tukey's test at 5%. Error bars represent the standard error of the means.

Table 1. Volatile organic compounds identified in broccoli shoots macerates by solid-phase microextraction gas chromatography mass spectrometry^a

	Compound	RI Exp ^b	RI Lit ^c	Sim (%) ^d	Dry macerate	Aqueous macerate	Exposed water
Alcohol							
1	Ethanol	...	...	96	+	...	+
2	3-Pentanol	693	685	88	+	+	+
3	3-Methyl-butanol	730	734	92	+	...	+
4	3-Hexenol (E)	849	851	95	++	...	+
5	3-Hexenol (Z)	855	857	92	++	...	+
6	Hexanol	869	869	91	++	...	...
7	Phenylethyl alcohol	1,110	1,116	88	+	...	...
Sulfuredes							
1	Metanethiol	...	...	91	+	+	...
2	Dimethyl sulfide	...	...	92	+	+	+
3	Methyl thiocyanate	709	711	91	+	+	+
4	Dimethyl disulfide	738	744	97	++	++	+
5	Dimethyl trisulfide	968	955	92	++	+	...
6	Dimethyl tetrasulfide	1,211	1,222	93	+	...	...
Ester							
1	3-Hexenyl pentanoate (E)	1,225	...	91	+	...	...
2	3-Hexenyl pentanoate (Z)	1,230	1,235	91	+	...	...
Aldehyde							
1	2-Heptenal	957	964	82	++	...	...
2	Nonanal	1,098	1,102	91	+	...	...
Ketone							
1	2-Nonanone	1,086	1,091	91	+	+	...
Others							
1	4-Methylthio butanenitrile	1,079	1,051	88	+	+	...
2	5-Methylthio entanenitrile	1,192	1,174	88	++	++	...

^a Symbols: + indicates presence of the molecule and ++ indicates present in a relatively higher concentration.

^b Calculated retention indices by injecting a homologous series of alkanes.

^c Theoretical retention index according to the literature (Adams 2007).

^d Similarity between the peak of the mass spectrum and mass spectrum of the library.

M. incognita J₂ mortality, with 98% after 72 h of exposure (Fig. 1A). The same period of exposure to VOC from broccoli shoots led to a 43% mortality for the dry macerate ($P < 0.01$), whereas the aqueous macerate just differed from the control at 72 h of exposure ($P = 0.02$) (Fig. 1B).

Infectivity (number of galls) and reproduction (number of eggs) were only tested for the sunflower seed macerate due to its higher activity on *M. incognita* J₂ mortality when compared with broccoli shoot macerates. The infectivity and reproduction of *M. incognita* decreased with the increment of the exposure period to sunflower seed macerates starting at 12 h of exposure ($P < 0.01$) and was similar for both dry and aqueous macerates. Reproduction was reduced by more than 80% and infectivity was reduced by 95% after 72 h of exposure (Fig. 1C and D).

Biofumigation with plant macerate against *M. incognita* in plastic cups. The immobility and mortality of *M. incognita* J₂ were higher when the nematode was exposed to VOC from sunflower seed macerates than to VOC from broccoli macerates ($P < 0.01$) (Fig. 2A

and B). VOC produced by the substrate mixed with sunflower seed macerates caused almost 100% immobility at a concentration of 2%, whereas VOC from broccoli shoot macerates showed a similar effect only at 4% (Fig. 2A). The mortality of J₂ exposed to 8% of broccoli and sunflower seed macerates mixed with the substrate was 46 and 64%, respectively (Fig. 2B).

Although there were differences in mobility and mortality of J₂ exposed to VOC (Fig. 1A and B), there were no significant differences between broccoli and sunflower seed macerates on the number of galls ($P = 0.62$) or on the number of eggs ($P = 0.90$) produced by *M. incognita* when the initial inoculum was eggs in direct contact with the biofumigated substrate (Fig. 2C and D). Broccoli and sunflower seed macerates incorporated into the substrate significantly reduced infectivity ($P < 0.01$) and reproduction ($P < 0.01$) of *M. incognita* in tomato roots, starting at 1% of macerate. At a concentration of 8% for both broccoli and sunflower, the number of galls and eggs per gram of roots was reduced by 89 and 95%, respectively, when compared with the 0% control (Fig. 2C and D).

Table 2. Volatile organic compounds identified in sunflower seed macerates by solid-phase microextraction gas chromatography mass spectrometry^a

	Compound	RI Exp ^b	RI Lit ^c	Sim (%) ^d	Dry macerate	Aqueous macerate	Exposed water
Alcohol							
1	Ethanol	...	...	98	++	...	+
2	3-Methyl-butanol	732	734	96	+	++	+
3	2-Methyl-butanol	736	738	92	+	...	+
4	2,3-Butanediol	819	...	96	+	++	...
5	1-Hexanol	869	869	92	+	+	+
6	2-Heptanol	904	905	94	...	++	...
7	2-Ethyl-hexanol	1,030	1,029	88	...	+	+
8	2-Nonanol	1,097	1,097	88	...	++	...
9	2-Undecanol	1,297	1,301	88	...	+	...
Terpenes							
1	α-Thujene	924	924	92	+	...	...
2	α-Pinene	932	932	94	++	+	...
3	Thuja-2,4(10)diene	952	953	80	...	+	++
4	Sabinene	972	969	81	+	...	...
5	β-Pinene	977	974	86	+	...	...
6	α-Terpinene	1,016	1,014	88	+	...	...
7	Ocimene	1,023	1,022	93	+	...	...
8	Limonene	1,027	1,024	92	+	...	...
9	Eucalyptol	1,030	1,026	91	+	...	...
10	γ-Terpinene	1,054	1,054	88	+	...	...
11	Transpinocar+eol	1,135	1,135	91	...	+	...
12	Verbenol (Z)	1,141	1,137	88	...	+	+
13	Terpienol	1,175	1,177	92	+	+	++
14	Mirtenol	1,192	1,193	90	...	+	+
15	Verbenone	1,203	1,204	86	+	...	+
Ester							
1	s-Methyl-thiopropionate	797	...	81	...	+	...
2	s-Metyl thioctanoate	1,291	1,293	91	...	+	...
Aldehyde							
1	3-Methyl-butanal	649	654	89	+	...	...
2	2-Methyl-butanal	658	664	92	+	...	...
Ketones							
1	2-Pentanone	684	685	94	...	+	+
2	2,3-Heptadione	832	...	95	...	+	...
3	2-Heptanone	888	889	91	...	++	+
4	3-Octanone	987	988	85	...	++	...
5	2-Nonanone	1,086	1,091	92	...	+	...
6	2-Undecanone	1,287	1,293	90	...	++	...
Phenol							
1	p-Vinyl guaiacol	1,307	1,309	92	...	+	...
2	Phenol	993	992	95	...	++	...
Others							
1	Acetic acid	702	...	97	+	...	+

^a Symbols: + indicates the presence of the molecule and ++ indicates present in a relatively higher concentration.

^b Calculated retention indices by injecting a homologous series of alkanes.

^c Theoretical retention index according to the literature (Adams 2007).

^d Similarity between the peak of the mass spectrum and mass spectrum of the library.

Effect of water exposed to plant VOC on *M. incognita*. Water exposed to VOC emitted by the macerate of broccoli shoots significantly lowered the mobility of *M. incognita* J₂ by 97% ($P < 0.01$), whereas water exposed to VOC from sunflower seed macerates did not affect the mobility of the J₂. The mortality of *M. incognita* J₂ exposed to VOC produced by broccoli and sunflower seed macerates was not significantly different from the control treatment ($P = 0.29$) (Fig. 3A). Increased time of exposure to VOC produced by broccoli shoot macerates significantly decreased the mobility of *M. incognita* ($P < 0.01$), with approximately 100% of the J₂ immobile after 24 h of exposure, whereas more than 83% of the J₂ remained motile in the control treatment (Fig. 3B). When water exposed to broccoli VOC for 1 or 2 days was applied to the infested soil, there were 98% fewer galls ($P < 0.01$) and 98% fewer eggs ($P < 0.01$) in tomato roots than in the water control (Fig. 3C).

Volatilome of plant macerates. Sunflower seed macerates produced an overall higher number of volatile molecules than broccoli shoot macerates (Tables 1 and 2). In general, a lower number of molecules was detected in the water exposed to dry macerates than in dry and aqueous macerates of both plant species. Twenty molecules were detected in dry broccoli shoot macerates and nine and eight molecules were identified in aqueous macerate and in the water exposed to dry macerates of broccoli, respectively. On the other hand, 19, 25, and 13 molecules were detected in dry and aqueous macerates and in water exposed to sunflower seed macerates, respectively. Sulfurated volatiles were exclusively produced by broccoli macerates, whereas terpenes, phenols, and acetic acid were only produced by sunflower seed macerates. Esters and aldehydes were present in dry macerates of broccoli, whereas ketones, alcohols, and sulfurated compounds were present in both dry and aqueous broccoli macerates. Aldehydes were only detected in dry sunflower seed macerates; esters, ketones, and phenols were found only in aqueous sunflower seed macerates and alcohols and terpenes were found in both dry and aqueous macerates of sunflower seed. As expected, all molecules detected in the water exposed to VOC were also detected in dry or aqueous broccoli or sunflower seed macerates. In water exposed to VOC emitted by broccoli macerate, only alcohols and sulfurated molecules were detected, whereas water exposed to sunflower seed macerates contained alcohols, ketones, terpenes, and acetic acid (Tables 1 and 2).

Activity of selected VOC from the broccoli volatilome. The three purified VOC used in this study were selected because they were detected only in the volatilome of broccoli, which produced VOC that were retained in water, making it toxic to nematodes, as

opposed to the VOC produced by sunflower seed. There was a significant effect of both 3-pentanol and DMDS on the immobility and mortality of *M. incognita* J₂ with increasing concentrations ($P < 0.01$) (Fig. 4) but not for DMS ($P = 0.48$). The concentration at which 50% of the J₂ were killed by the VOC (LC₅₀) calculated by Probit analyses was 176 ppm (upper limit = 129 and lower limit = 240) for DMDS and 918 ppm (upper limit = 993 and lower limit = 849) for 3-pentanol.

The water exposed to DMDS, 3-pentanol, and the mixture of DMDS + 3-pentanol + DMS caused, on average, 93% immobility and 78% mortality in J₂ of *M. incognita*, higher than the control and DMS, where immobility was approximately 1 to 2% ($P < 0.01$) (Fig. 5A and B). DMS-exposed water did not affect the mobility and viability of *M. incognita* as compared with the control (Fig. 5). When the J₂ suspension along with water exposed to DMDS, 3-pentanol, or the mixture of the three VOC was applied on the soil, there was a reduction in the number of galls and eggs that averaged 65 and 93.8%, respectively ($P < 0.01$). In general, DMDS had a stronger activity than 3-pentanol, because DMDS resulted in higher J₂ mortality ($P < 0.01$) (Fig. 5B) and a stronger reduction in the number of eggs ($P < 0.01$) (Fig. 5C and D).

Discussion

Broccoli shoot and sunflower seed macerates produced VOC that increased the mortality and decreased mobility, infectivity, and reproduction of *M. incognita*. In general, dry macerates were more effective in killing *M. incognita*, particularly in the case of broccoli. However, dry and aqueous macerates of sunflower seed were equally effective in decreasing infectivity and reproduction of this nematode (Fig. 1). The water in the aqueous macerates may be responsible for retaining the VOC produced by broccoli and, consequently, for the lower emission of VOC from this type of macerate.

Even though sunflower seed macerates were more efficacious than broccoli macerates in decreasing mobility and increasing mortality of J₂ in biofumigation experiments (Fig. 2), only the water exposed to broccoli showed activity against *M. incognita* (Fig. 3). The water does not retain the VOC that make the sunflower seed macerates toxic to nematodes but does retain some of the VOC from broccoli macerates that were originally less toxic than sunflower seed macerates (Fig. 4). Alcohols and sulfurated VOC are responsible for the toxicity of the water exposed to broccoli macerates. The toxic VOC from sunflower seed were not retained in the water. The retention of the VOC in water depends on many parameters such as boiling point, polarity, and their concentration in the gas phase. However,

Fig. 4. Purified volatile organic compounds (VOC) from the broccoli shoot volatilome against *Meloidogyne incognita* second-stage juveniles (J₂). **A**, Immobility and mortality of J₂ exposed to dimethyl disulfide at different concentrations. **B**, Immobility and mortality of J₂ exposed to different concentrations of 3-pentanol. The J₂ were exposed to the different concentrations of the VOC for 48 h before the evaluations. Error bars represent the standard error of the means. LC₅₀ indicates lethal concentration required to kill 50% of the J₂ calculated by Probit analysis.

as can be observed in Table 2, with the exception of thuja-2,4(10) diene, all compounds retained in water present an OH or CO in their chemical structures, which can promote hydrogen bonding with water molecules (Tainter et al. 2012) and, therefore, increase retention in the exposed water. Consequently, all VOC from sunflower seed that are retained in water may be classified as nontoxic to *M. incognita*.

The significant reduction in infectivity and reproduction observed in *M. incognita* J₂ exposed to VOC produced by sunflower, broccoli, and the water exposed to broccoli may be due to paralysis of the J₂ rather than to their mortality (Figs. 2 and 3). VOC produced by plants usually do not cause high mortality but often are responsible for immobilizing the *M. incognita* J₂, which would explain the low infectivity and reproduction in tomato roots. This confirms our previous results with VOC from macerates of mustard and neem leaves against *M. incognita* (Barros et al. 2014a,b). Apparently, toxic VOC may affect the nervous system of the nematodes, which disturbs the ability of the J₂ to locate the host or develop feeding sites (Baillie and Wright 1985; Gentry et al. 2013).

The biofumigation experiments indicated that VOC may act alone or synergistically with non-VOC to reduce the viability of *M. incognita*,

which also has been observed by other authors working on diverse green residues (Bailey and Lazarovits 2003; Estupiñán-López et al. 2017; Lord et al. 2011; Neves et al. 2007; Ploeg 2008; Ploeg and Stapleton 2001; Roubtsova et al. 2007; Wen et al. 2013). Biofumigation is traditionally performed with *Brassica* spp., which produce VOC and non-VOC with toxic effect against plant-parasitic nematodes (Aissani et al. 2015; Al-Rehiayani et al. 1999; Neves et al. 2007; Ngala et al. 2015; Roubtsova et al. 2007). However, despite the fact that sunflower seed non-VOC has already presented nematocidal or fungicidal activities (Ganaie et al. 2011; Giudici et al. 2000; Regente and De La Canal 2000), our study showed that sunflower is also able to produce VOC toxic to *M. incognita* and has the potential to be used in biofumigation.

VOC produced by broccoli macerates turned the water toxic to *M. incognita* in less than 24 h of exposure. In previous studies (Barros et al. 2014b; Grimme et al. 2007; Silva et al. 2017; Terra et al. 2017, 2018), the minimum exposure time to turn the water toxic to nematodes was not determined with precision. Due to their ability to become trapped in water, VOC generated during biofumigation may be employed in combination with irrigation practices to control nematodes. VOC retained in water are possibly active at long

Fig. 5. Effect of water exposed to purified volatile organic compounds (VOC) from the broccoli shoot volatilome on *Meloidogyne incognita*. **A**, Immobility and **B**, mortality of second-stage juveniles (J₂) treated with water exposed to 150 μ l of purified VOC or to a mixture of VOC containing 50 μ l of each. **C**, Number of galls and **D**, number of eggs in tomato root inoculated with J₂ treated with water exposed to 150 μ l of purified VOC or to a mixture of 50 μ l of each VOC. Numbers of galls and eggs were determined 45 days after the inoculation of 500 J₂ on tomato roots (*Solanum lycopersicum* 'Santa Clara'). DMDS = dimethyl disulfide, DMS = dimethyl sulfide, and Mixture = DMS + DMDS + 3-pentanol. Means followed by the same letter are not significantly different according to Tukey's test at 5% probability. Error bars represent the standard error of the means.

distances from their site of release. Water is able to penetrate the soil pores (Wheatley 2002) and the VOC retained in it may turn the soil solution toxic to nematodes. It would be interesting to perform studies to determine the distance from the point of release and the length of time that the water remain toxic to nematodes, because VOC will eventually dissipate from the solution.

Although we are still not certain about the identity of all compounds responsible for the significant activity of broccoli shoot macerates and the exposed water to *M. incognita*, sulfurous compounds are certainly involved. Sulfurous compounds such as isothiocyanates are known nematicides produced during biofumigation with *Brassica* spp. (Lord et al. 2011). However, the toxicity of *Brassica* spp. against nematodes involves diverse molecules (Vervoort et al. 2014). DMDS and methyl thiocyanate are sulfurous compounds identified in the water exposed to broccoli macerates and were previously reported to control plant-parasitic nematodes (Zasada and Ferris 2003). DMDS is a commercial molecule (Paladin EC; Arkema Inc., Comblombes, France) already used to control *M. incognita* (Kokalis-Burelle et al. 2016). A third sulfurous volatile, DMS, also identified in the water exposed to broccoli macerates, did not show any activity against *M. incognita* even at the highest concentration tested (1,100 ppm). These observations combined with the results of the purified compounds (Fig. 3) confirm that DMDS and methyl thiocyanate are involved in the toxicity of broccoli to *M. incognita* in soil biofumigation or when its VOC are retained in water. Purified methyl thiocyanate was not tested in our study but it appears to be more toxic to *M. incognita* than DMDS, with a reported LC₅₀ of 18 ppm (Aissani et al. 2015), as compared with 176 ppm for DMDS (this study). The LC₅₀ of these compounds is close to that of the commercial nematicide Carbofuran, which is 191 ppm (Terra et al. 2018), making methyl thiocyanate a potential compound for commercial development and field application as DMDS.

Some alcohols found in the volatilome of broccoli and sunflower seed have been shown to be toxic against plant pathogens (Barros et al. 2014b; Fialho et al. 2010; Silva et al. 2017). Among these, 3-pentanol was retained in water immobilizing and killing the *M. incognita* J₂ but only at high concentrations (LC₅₀ = 918 ppm). No synergistic effect was observed when the three purified compounds (DMS + DMDS + 3-pentanol) were applied together. However, some plant VOC act in concert to inhibit *Meloidogyne* spp. (Ntalli et al. 2011; Silva et al. 2013). Compounds such as 2,3-butanediol, sabinene, eucalyptol, limonene, and α -thujene found in sunflower seed emission were also reported in other volatilomes with activity against *M. incognita* (Barros et al. 2014b; Gonçalves et al. 2016; Silva et al. 2013) but were not retained in the water.

Broccoli shoots and sunflower seed have a great potential to be used in biofumigation against *Meloidogyne* spp. and should be more exploited as intercrops, companion crops, or green manures for field application. Although broccoli and sunflower are not hosts for *M. incognita*, broccoli hosts other root-knot nematode species (Ciancio and Mukerji 2008). To avoid the build-up of these species on broccoli, one possible strategy would be to grow broccoli during the cooler seasons when nematode activity is limited and reproduction is slow (Ploeg 2008). Another interesting application of these plant species could be in soil incorporation combined with irrigation and soil tillage (Dutra and Campos 2003). The humidity of irrigation would stimulate the eggs to hatch and the absence of host plants combined with soil tillage would make the J₂ deplete their energy reserves without finding a suitable host. Additionally, these conditions combined with the toxic effects of the VOC released from the incorporated plants may further decrease the viability of the nematode.

Despite the fact that the plant parts used in this study are the commercial products of these crops, a small portion of the production could be used as green manure once a volume of approximately 2 to 4% (wt/wt) was enough to significantly reduce nematode infection and reproduction. A generally recommended amount for plant incorporation as biofumigant is 50 t/ha (about 2.5%), although the efficacy of materials can vary depending on the soil and plant properties and the method of application (Bello et al. 2002; Ploeg 2008). Similar amounts of *Brassica* spp. have been used under field conditions in

biofumigation to control the root-knot nematode *M. chitwoodi* in potato production (Mojtahedi et al. 1993). In large production fields, this volume is unfeasible for commercial parts of the plant; however, in small horticulture areas or greenhouses, this amount may be smaller than the residue that remains in the area or is discarded due to its low quality for commercial use. Particularly in the case of broccoli, approximately 6 to 10% of the shoots are discarded in small farms (Bekhit et al. 2013; Gustavsson and Stage 2011). Thus, biofumigation has shown to be more efficient in small production areas (Ploeg 2008) and the application of these crop residues in such areas may be feasible as biofumigants.

In summary, we showed that VOC from both broccoli shoots and sunflower seed have nematicidal activity against *M. incognita* in vitro and in biofumigation along with non-VOC. Therefore, these VOC should be more exploited as nematicidal compounds. VOC from both plant species were equally effective in decreasing infectivity and reproduction of the nematode but only the water exposed to broccoli macerates was toxic to *M. incognita*. Furthermore, the compounds DMDS and 3-pentanol are toxic to *M. incognita* with LC₅₀ of 176 and 918 ppm, respectively, and can be retained in exposed water, showing their potential to be used as nematicidal compounds.

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