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ORIGINAL PAPER

Attraction behaviors of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) to synthetic volatiles emitted by insect-damaged carrot roots

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Abstract Entomopathogenic nematodes (EPNs) play a role in indirect defenses of plants under attack by root herbivores. We have tested the chemotactic responses of 4 EPN species (*Steinernema feltiae*, *S. carpocapsae*, *S. kraussei*, and *Heterorhabditis bacteriophora*) to 5 compounds ([1] α-Pinene, [2] Terpinolene, [3] Bornyl acetate, [4] 2-Ethyl-hexanol, and [5] 2, 4-Di-tert-butylphenol) released by damaged (3, 4, 5) and undamaged (1, 2) carrot roots. We hypothesized that the EPN directional responses to the tested volatile compounds (VOCs) could be related to foraging strategy and would vary among species, VOC, and VOC concentrations. Our results indicate that all of the tested EPN species exhibited a weak attraction or repulsion to volatiles, irrespective of their foraging strategy. Terpinolene was a repellent for EPN species classified in all three foraging groups. However, such values of chemotaxis index (CI) were reported with EPN species only when pure concentration of VOC was used. Based on our current results, we conclude that responses to distinct volatile cues are a species-specific characteristic. Our results suggest that EPNs are able to distinguish herbivore-induced chemicals from chemicals that are typical for healthy roots.

Keywords Indirect plant defense · Chemosensation · Entomopathogenic nematodes · Terpinolene

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Key message

- VOCs have an important role in the tritrophic system consisting of a plant, a herbivore, and its natural enemy.
- The chemotactic responses of four EPN species to five compounds released by damaged and undamaged carrot roots are reported.
- Our results suggest that responses to distinct volatile cues are a species-specific characteristic and irrespective of their foraging strategy.
- Current results suggest that EPNs are able to distinguish herbivore-induced chemicals from chemicals that are typical for healthy roots.

Introduction

Tritrophic interactions involving plants, herbivores, and parasites have been documented for belowground systems, where entomopathogenic nematodes (EPNs) can exploit root herbivore-induced volatile compounds to locate their hosts (Rasmann et al. 2005; Rasmann and Turlings 2008; Ali et al. 2010). Soil is the natural habitat of EPNs from the families Steinernematidae and Heterorhabditidae, and their application in pest management has been primarily against soil-dwelling insect pests (Ishibashi and Choi 1991; Kaya and Gaugler 1993; Koppenhöffer et al. 2004). In both *Steinernema* and *Heterorhabditis*, there is a single free-living stage, the infective juvenile (IJ), that carries in its gut bacteria of the genus *Xenorhabdus* and *Photorhabdus*, respectively (Boemare et al. 1993). On encountering a suitable insect, the IJ enters through the mouth, anus, or spiracles and makes its way to the haemocoel (Eidt and Thurston 1995). Some species may also penetrate through

the intersegmental membranes of the insect cuticle (Peters and Ehlers 1994). In the haemocoel, the IJ releases cells of its bacterial symbiont. Bacteria multiply rapidly in hemolymph and produce toxins, which contribute to the weakening of the host's defense mechanism. The host attacked by EPN usually dies because of poisoning or failure of certain organs in 24 to 72 h after the infection (Forst and Clarke 2002).

The behavior of EPN has been intensively studied and different EPN species behave very differently in terms of dispersal and host-finding (Lewis 2002; Campbell et al. 2003). The ability of EPN IJ to disperse actively through soil and locate a host is a key element for the success of application of certain EPN species in pest management. IJ host-finding strategies differ from species to species (Lewis 2002; Campbell et al. 2003). Foraging strategies used by IJs to find a host vary between cruise (*Heterorhabditis bacteriophora*, and *Steinernema kraussei*), intermediate (*S. feltiae*), and ambush (*S. carpocapsae*) (Lewis 2002; Campbell et al. 2003). However, researches on their behavior have not considered the natural habitat of these nematodes. Kruitbos et al. (2009) suggested that EPNs may be habitat specialists and highlighted the difficulties of studying soil-transmitted parasites in non-soil media.

The rhizosphere provides a very attractive environment for a vast number of organisms (Wenke et al. 2010). Root exudates are chemically diverse, beginning with compounds such as amino acids and amides, organic acids, phenols, sugars, as well as a wide variety of secondary metabolites, polysaccharides, and proteins of higher molecular mass (Wenke et al. 2010). However, volatile compounds (VOCs) can also be detected in the rhizosphere of several plant species (Bais et al. 2004; Bais et al. 2006; Rasmann et al. 2005; Erb et al. 2013; Hiltpold et al. 2013). Ali et al. (2010) have demonstrated that citrus roots upon feeding by the root weevil *Diaprepes abbreviatus* emit several terpenes in the surrounding soil. Rasmann and Turlings (2008) reported that roots of cotton (*Gossypium herbaceum*) after feeding by the larvae of the chrysomelid beetle *Diabrotica balteata* emit >10 compounds, among which at least seven terpenoid volatiles were observed. Rasmann et al. (2005) reported that maize roots damaged by larvae of *Diabrotica virgifera virgifera* [Coleoptera: Chrysomelidae, known commonly as western corn rootworm (WCR)] emit a key attractant for EPNs. The compound in question (E)-β-caryophyllene proved to be a weak attractant for *H. megidis*, one of the most infectious nematode against WCR. Volatile metabolites emitted underground enable plants to directly and indirectly influence the community of soil-dwelling organisms (Bais et al. 2006; Erb et al. 2013). Using volatile metabolites plants can defend themselves against herbivores and plant pathogenic fungi and bacteria, support beneficial symbiosis, and combat competitive plant species (Bais et al. 2006). Chemotaxis is the main sensory mode nematodes use to orient themselves to their hosts. IJs have been shown to respond to both CO_2 and other cues (Hallem et al. 2011; Dillman et al. 2012; Turlings et al. 2012). There are reports that IJs move toward or away from host excretory products, changes in pH, temperature, bacterial symbionts, electrical fields, and various plant volatile compounds (Burman and Pye 1980; Grewal et al. 1993; Rasmann et al. 2005; Shapiro-Ilan et al. 2012).

Wireworms, the soil-dwelling larval stages of the click beetle (Coleoptera: Elateridae), are a serious pest problem worldwide (Kuhar and Alvarez 2008). As polyphagous insects, they attack a number of important crops (e.g., potato, carrots, sugar beet, and occasionally cereals) (Parker and Howard 2001). White grubs are the root-feeding larvae of scarab beetles (Coleoptera: Scarabaeidae), and they are among the most destructive pests of horticultural plants, pastures, and turfgrass in many parts of the world (Laznik and Trdan 2015). Both species damage crops by feeding on their root systems after planting, which can significantly reduce crop quality (Jackson and Klein 2006; Johnson et al. 2008).

Here, we describe our study of the chemotactic behavior of *Steinernema feltiae* (Filipjev), *Steinernema carpocapsae* Weiser, *Steinernema kraussei* (Steiner), and *Heterorhabditis bacteriophora* Poinar toward α-Pinene, Bornyl acetate, 2, 4-Di-tert-butylphenol, 2-Ethyl-hexanol, and Terpinolene; VOCs released from insect (wireworms and grubs)-damaged carrot roots (Weissteiner and Schütz 2006; Weissteiner 2010). The aims of our research were (1) to study the effect of different EPN foraging strategies (ambush, intermediate, or cruise) toward the tested VOCs (2) to determine whether chemotaxis is species specific, and (3) to assess whether the VOCs from damaged and undamaged carrot roots have any effect on the tested EPNs behavior, and (4) if these VOCs are a part of an indirect plant defense.

Materials and methods

Source and maintenance of entomopathogenic nematodes

Four EPN species were tested in the experiment. The commercial preparations of Nemasys (a.i. *S. feltiae*), Nemasys C (a.i. *S. carpocapsae*), Nemasys L (a.i. *S. kraussei*), and Nemasys G (a.i. *H. bacteriophora*) were obtained from Becker Underwood. All EPN species were reared using the last instar larvae of *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) (Bedding and Akhurst 1975). *G. mellonella* was reared in a controlled environment at 28 ± 2 °C, 60 % relative humidity (RH) with a 12-h photoperiod (Woodring and Kaya 1988; Parra 1998). The IJs were stored at 4 °C at a density of 2000 IJ ml^{-1}. We

only used IJs that were less than 2-week old (Laznik and Trdan 2013). The concentration of the EPN suspension was calculated according to Laznik et al. (2010). Nematode viability was determined prior to initiation of the chemotaxis experiment (Laznik and Trdan 2013), and only nematode stocks with >95 % survival were used (De Nardo and Grewal 2003).

Tested volatile compounds

The choice of VOCs used in our investigation was based on the research of Weissteiner (2010). Organically cultivated carrots (*Daucus carota* ssp. *sativus*) were used in their investigation. Larvae of *Melolontha hippocastani* and *Agriotes* sp. were used in their experiment in order to damage carrot roots. Gas chromatography–Mass spectrometry (GC–MS) analysis of VOCs released by undamaged and damaged roots was used in order to show different feeding-induced volatile pattern when infested by *Melolontha* or *Agriotes* larvae. Results of their investigation showed that undamaged carrot roots release several VOCs and among them were also (1) α-Pinene and (2) Terpinolene. Wireworm-damaged carrot roots release (3) Bornyl acetate and (4) 2-Ethyl-hexanol. Grub-damaged carrot roots release VOCs (3), (4), and (5) 2, 4-Di-tert-butylphenol. In order to perform our investigation, we used synthetic-produced compounds (Sigma Aldrich). The VOCs in the experiment were tested at two concentrations: (1) at pure concentration (O'Halloran and Burnell 2003; Laznik and Trdan 2013) and (2) at 0.03 ppm (the average concentration of VOCs in soil, 10 cm from the root system) (Weissteiner et al. 2012).

Chemotaxis assay

The chemotaxis assay was based on an assay developed by Ward (1973) and O'Halloran and Burnell (2003) and modified by Laznik and Trdan (2013). The assay plates used were Petri dishes, 9 cm in diameter containing 25 ml of 1.6 % technical agar (Biolife, Milano, Italy), 5 mM potassium phosphate (pH 6.0), 1 mM $CaCl_2$, and 1 mM $MgSO_4$. Three circular marks (1 cm in diameter) were made on the bottom of the plate: first in the center, then on the right and lastly on the left side of the Petri dish, 1.5 cm from its edge. A 10 µl drop of tested substance was placed on the right side of the agar surface (treated area), and 10 µl of distilled water (control area) (Laznik and Trdan 2013) was placed on the left side of the agar surface (both parts represent outer circles). The VOCs were immediately applied to the agar plates before the application of the nematodes (Bargmann and Horvitz 1991). A 50 µl drop of 100 IJs was placed in the center of the agar surface (inner circle). In control treatment 10 µl of distilled water was applied in control and treated area, and a 50 µl drop of 100 IJs was placed in the center of the agar surface. Each treatment included five replicates. All of the experiments were repeated 3 times. The Petri dishes were placed in a rearing chamber (RK-900 CH, Kambič Laboratory equipment, Semič, Slovenia) at 22 °C and 75 % RH, without light. The nematodes were allowed to move freely for 2 or 24 h, and the Petri dishes were then placed in a freezer at −20 °C for 3 min to immobilize the nematodes. The number of nematodes in the treatment and control areas was counted using a binocular microscope (Nikon C-PS) at ×25 magnification. The specific chemotaxis index (CI) (Bargmann and Horvitz 1991) was calculated as follows:

$$\left(\text{Number of nematodes in the treatment area} - \text{Number of nematodes in the control area}\right) / \text{Total number of nematodes in the assay}$$

The CI varied from 1.0 (perfect attraction) to −1.0 (perfect repulsion). In the experiments reported here, compounds with a CI are classified as follows: ≥0.2, as attractive; from 0.2 to 0.1, as a weak attractant; from 0.1 to −0.1, no effect; from −0.1 to −0.2, as a weak repellent and ≤−0.2, as a repellent to EPNs (Laznik and Trdan 2013).

Statistical analysis

For all of the treatments and controls, preferential movement of nematodes from the inner to the outer circle of the Petri dish (i.e., a directional response) was determined using a paired *t* test comparing the number of IJs in the inner versus the outer circle (Statgraphics Plus for Windows 4.0; Shapiro-Ilan et al. 2012; $\alpha = 0.05$). Additionally, to compare response levels among the foraging strategies, the average number of IJs that moved to the outer circle or stayed in the inner circle was calculated for each dish, and average numbers were compared through an analysis of variance (ANOVA, $\alpha = 0.05$). Additionally, an analysis of variance (ANOVA) was performed on the CI to compare the level of response to the tested volatile compounds among the different EPN species depending on the exposure time and concentration, the means were separated by Duncan's multiple range test with a significant level of $p < 0.05$. The data are presented as the mean ± SE. All of the statistical analyses were performed using Statgraphics Plus for Windows 4.0 (Statistical Graphics Corp., Manugistics, Inc., Rockville, MD, USA).

Results

Diversity of movement among EPN species and their foraging strategies

Analysis of the results showed that directional movement in response to volatile compounds from the inner (central

part of the petri dish) to outer test circles (control and treated area) was influenced by different factors and their interactions (Table 1). Based on the t test results ($t = 56, 73$; $p < 0.0001$; $\alpha = 0.05$), statistically significant differences were observed among the average number of IJs in the inner (83.0 ± 1.2) and outer (17.0 ± 0.6) circles after 24 h. There were significant differences in the average number of IJs in the outer circles among ambushers (12.9 ± 1.4), intermediates (16.4 ± 1.3), and cruisers (23.1 ± 0.9). Furthermore, after only 2 hours, an average of 3.1 ± 1.0 IJs moved to the outer circle, whereas after 24 h an average of 31.8 ± 2.1 IJs moved. Among the tested concentrations, there was a significantly higher number of IJs in the outer circles at pure concentration (23.5 ± 1.3) while an average of only 11.4 ± 0.8 IJs moved at 0.03 ppm. In the terpinolene treatment, we found a significantly higher number of EPNs in the outer circles (16.3 ± 1.2). There were also differences among cruisers (21.5 ± 1.0), intermediates (14.9 ± 0.5), and ambushers (12.6 ± 0.7) in movement toward the outer circles in the Terpinolene treatment. Foraging strategy did not affect the movement of IJs toward the other tested volatile compounds or the control. Among the tested EPN species, a significantly higher number of IJs in the outer circles was confirmed for *S. kraussei* (29.4 ± 0.2) and *H. bacteriophora* (29.2 ± 1.3). The number of IJs in the outer circles was significantly lower for *S. feltiae* (20.8 ± 2.4) and *S. carpocapsae* (16.3 ± 3.0).

Chemotaxis index

The analyses of the results showed that CI values were influenced by the species of EPN ($F = 3.62$; df = 3, 478; $p = 0.0131$), concentration of the volatile compound ($F = 6.84$; df = 1, 478; $p = 0.0092$), volatile compound ($F = 2.58$; df = 6, 478; $p = 0.0183$), time of exposure ($F = 12.62$; df = 1, 478; $p = 0.0004$), and interaction between EPN species and time of exposure ($F = 9.21$; df = 7, 478; $p < 0.0001$). Foraging strategy ($F = 0.50$; df = 2, 478; $p = 0.6055$); interaction between volatile compounds and foraging strategy ($F = 0.79$; df = 12, 478; $p = 0.6571$); interaction between volatile compounds and time of exposure ($F = 1.03$; df = 6, 478; $p = 0.4071$); and interaction between volatile compounds, foraging strategy, and time of exposure ($F = 0.90$; df = 12, 478; $p = 0.5462$) did not have a statistically significant influence on the CI values.

None of tested EPNs in our investigation showed any behavior response to tested VOCs at a concentration of 0.03 ppm after 2 h (Table 2). IJs of *S. feltiae*, *S. carpocapsae* did not show any behavior response to tested VOCs at a concentration of 0.03 ppm after 24 h (Table 3). The analysis of the CI values of different VOCs after 24 h at a concentration of 0.03 ppm showed that 2, 4-Di-tert butylphenol was a weak repellent (CI = −0.15 ± 0.03) for *S. kraussei* (Table 3). For other cruisers nematode species in our investigation (*H. bacteriophora*), the same VOC proved to be a

Table 1 ANOVA results for the directional movement of IJs from the inner to the outer circle

Source	F	df	p
Foraging strategy	19.71	2	<0.0001[a]
Species	37.41	3	<0.0001[a]
VOC	2.13	5	0.0407[a]
Time of exposure	357.47	1	<0.0001[a]
Concentration of VOC	68.76	1	<0.0001[a]
Temporal replication	0.42	4	0.9041
Spatial replication	0.48	2	0.8221
Foraging strategy × VOC	0.78	10	0.6692
Foraging strategy × time of exposure	5.94	2	0.0029[a]
Foraging strategy × concentration of VOC	43.51	2	<0.0001[a]
VOC × time of exposure	2.53	5	0.0204[a]
VOC × concentration of VOC	0.98	5	0.4457
VOC × species	1.08	15	0.3777
Species × time of exposure	0.76	3	0.6874
Species × concentration of VOC	2.01	3	0.0578
Foraging strategy × VOC × concentration of VOC	1.18	10	0.3002
Foraging strategy × VOC × time of exposure	1.04	10	0.4065
Species × VOC × concentration of VOC	0.91	15	0.6100
Species × VOC × time of exposure	0.77	15	0.6821

[a] The source of variation was significant at $\alpha = 0.05$

weak attractant (CI = 0.17 ± 0.07). Similar findings were confirmed also in response of *H. bacteriophora* to α-Pinene (CI = 0.18 ± 0.07) (Table 3). Terpinolene proved to be a weak repellent (CI = −0.17 ± 0.07) of *H. bacteriophora* after 24 h, at a concentration of 0.03 ppm (Table 3). IJs of *S. feltiae*, *S. carpocapsae*, and *H. bacteriophora* did not show any behavior response to tested VOCs at pure concentration after 2 h (Table 4). The analysis of the CI values of different VOCs after 2 h at pure concentration showed that Terpinolene was a repellent (CI = −0.21 ± 0.02) for *S. kraussei* (Table 4). VOC α-Pinene, at a pure concentration after 24 h, proved to be a weak repellent for *S. carpocapsae* (CI = −0.11 ± 0.03), *S. kraussei* (CI = −0.14 ± 0.02), and *H. bacteriophora* (CI = −0.10 ± 0.02) (Table 5). Furthermore, VOCs 2-Ethyl-1-hexanol (CI = −0.11 ± 0.06) and Bornyl acetate (CI = −0.16 ± 0.04) proved to be a weak repellents for *S. carpocapsae* in our investigation (Table 5). In a contrast, VOC Bornyl acetate was a weak attractant (CI = 0.13 ± 0.05) for *S. kraussei* in our investigation at a pure concentration after 24 h (Table 5). The analysis of the CI values of different VOCs after 24 h at a pure concentration showed that Terpinolene was a repellent for *S. carpocapsae* (CI = −0.23 ± 0.04) and *S. feltiae* (CI = −0.23 ± 0.03) (Table 5).

Discussion

Our results show that the chemosensation of IJs toward and away from insect-induced carrot root volatile compounds (Weissteiner and Schütz 2006; Weissteiner 2010) varied depending on the EPN species, volatile compound, concentration of volatile compound, time of exposure, and interaction between EPN species and time of exposure. Our results indicate that all tested EPN species exhibited very low chemotaxis to volatiles irrespective of their foraging strategy. The highest value of CI −0.23 ± 0.03) was in our investigation reported when the IJs of *S. feltiae* were exposed to Terpinolene. In several related studies (O'Halloran and Burnell 2003; Hallem et al. 2011; Dillman et al. 2012), authors report many CIs above 0.5 for *H. bacteriophora* and *S. carpocapsae*, species which were included also in our investigation. In a related research (Laznik and Trdan 2013), authors used the same strain of EPN species as in our current investigation. The values of CI of EPNs toward β-caryophyllene, linalool, and α-caryophyllene were similar low as in our current investigation toward other VOCs. One possible explanation of low chemotaxis to volatiles can be a strain-specific characteristic of EPNs. Laznik and Trdan (2013) suggested that the response to different volatile cues is more a strain-specific characteristic than a different host-searching strategy. Since the strains used in other related studies (O'Halloran and Burnell 2003; Hallem et al. 2011; Dillman et al. 2012) were different to ours, we could confirm our previous results. The second possible explanation of low chemotaxis to volatiles in our investigation in comparison with related studies can be explained with the use of different VOCs as were used in our current study. Anyway, our results demonstrate that EPNs have evolved specialized olfactory system that is able to distinguish herbivore-induced chemicals from chemicals that are typical for healthy carrot roots. Similar conclusions were also reported in the recent research from Ali et al. (2011), in which the cruiser *Heterorhabditis indica* (Lewis 2002), the ambusher *S. carpocapsae* (Lewis 2002), and the intermediate *S. diaprepesi* and *S. riobrave* (Lewis 2002) were all attracted to root weevil *Diaprepes abbreviatus*-damaged roots of the Swingle rootstock. Our current results suggest that responsiveness to different volatile cues is a species-specific characteristic.

In our investigation, Terpinolene was a repellent for EPN species classified in all three foraging groups. However, such values of CI were reported with the EPN species *S. feltiae*, *S. kraussei*, and *S. carpocapsae* only when pure concentration of VOC was used. Off course, such high concentration of VOCs is unrealistic (Köllner et al. 2004) and probably toxic to the EPNs. With lower concentration of VOCs (0.03 ppm), which is the average concentration of VOCs found in soil, 10 cm away from the root system

Table 2 Effect of different VOCs on the chemotactic response of the EPN species, at a concentration of 0.03 ppm after 2 h

	2-Ethyl-1-hexanol	Bornyl acetate	2, 4-Di-tert-butylphenol	Terpinolene	α-Pinene	Control
Sc	0 ± 0Aa	0 ± 0Aa	0 ± 0Aa	0 ± 0Aa	0 ± 0Aa	0 ± 0Aa
Sf	0 ± 0Aa	0 ± 0Aa	0 ± 0Aa	0 ± 0Aa	0 ± 0Aa	0 ± 0Aa
Sk	0 ± 0Ab	−0.01 ± 0.01Ab	−0.01 ± 0.03Aab	−0.03 ± 0.02Ba	0 ± 0Ab	0 ± 0Ab
Hb	0 ± 0Aa	0 ± 0Aa	0 ± 0Aa	0 ± 0Aa	0 ± 0Aa	0 ± 0Aa

Each data point represents the mean CI ± SE. Each data with the same letter are not significantly different ($p > 0.05$). The capital letters indicate statistically significant differences among different EPN species and same VOC. The small letters indicate statistically significant differences among different VOCs within the same EPN species

Sc, *Steinernema carpocapsae*; *Sf*, *Steinernema feltiae*; *Sk*, *Steinernema kraussei*; *Hb*, *Heterorhabditis bacteriophora*; *Ambusher*, *S. carpocapsae*; *Cruiser*, *S. kraussei* and *H. bacteriophora*; *Intermediate*, *S. feltiae*

Table 3 Effect of different VOCs on the chemotactic response of the EPN species, at a concentration of 0.03 ppm after 24 h

	2-Ethyl-1-hexanol	Bornyl acetate	2,4-Di-tert-butylphenol	Terpinolene	α-Pinene	Control
Sc	0.04 ± 0.02Bb	0.05 ± 0.0 Bb	0.04 ± 0.02Cb	0 ± 0Ca	0 ± 0.03Aab	−0.02 ± 0.03Aa
Sf	−0.05 ± 0.03Aa	0 ± 0Ab	−0.09 ± 0.02Ba	−0.04 ± 0.02Ba	−0.02 ± 0.02Aab	−0.06 ± 0.02Aa
Sk	0.06 ± 0.04Bc	−0.1 ± 0.02Aab	−0.15 ± 0.03Aa	0.03 ± 0.02Dc	−0.06 ± 0.03Ab	−0.07 ± 0.02Ab
Hb	−0.09 ± 0.06Aab	0.01 ± 0.07ABb	0.17 ± 0.07Dc	−0.17 ± 0.07Aa	0.18 ± 0.07Bc	−0.01 ± 0.04Ab

Each data point represents the mean CI ± S.E. Each data with the same letter are not significantly different ($p > 0.05$). The capital letters indicate statistically significant differences among different EPN species and same VOC. The small letters indicate statistically significant differences among different VOCs within the same EPN species

Sc, *Steinernema carpocapsae*; *Sf*, *Steinernema feltiae*; *Sk*, *Steinernema kraussei*; *Hb*, *Heterorhabditis bacteriophora*; Ambusher, *S. carpocapsae*; Cruiser, *S. kraussei* and *H. bacteriophora*; Intermediate, *S. feltiae*

Table 4 Effect of different VOCs on the chemotactic response of the EPN species, at pure concentration after 2 h

	2-Ethyl-1-hexanol	Bornyl acetate	2,4-Di-tert-butylphenol	Terpinolene	α-Pinene	Control
Sc	0 ± 0Ba	0 ± 0Ba	0 ± 0Aa	0 ± 0Ba	0 ± 0Ba	0 ± 0Aa
Sf	0 ± 0Ba	0.05 ± 0.02Cb	0.01 ± 0.01Aa	0 ± 0Ba	0.02 ± 0.02Bab	0 ± 0Aa
Sk	−0.08 ± 0.03Abc	−0.03 ± 0.02Ac	−0.02 ± 0.03Ac	−0.21 ± 0.02Aa	−0.09 ± 0.01Ab	0 ± 0Ac
Hb	0 ± 0Ba	0 ± 0Ba	0 ± 0Aa	0 ± 0Ba	0 ± 0Ba	0 ± 0Aa

Each data point represents the mean CI ± S.E. Each data with the same letter are not significantly different ($p > 0.05$). The capital letters indicate statistically significant differences among different EPN species and same VOC. The small letters indicate statistically significant differences among different VOCs within the same EPN species

Sc, *Steinernema carpocapsae*; *Sf*, *Steinernema feltiae*; *Sk*, *Steinernema kraussei*; *Hb*, *Heterorhabditis bacteriophora*; Ambusher, *S. carpocapsae*; Cruiser, *S. kraussei* and *H. bacteriophora*; Intermediate, *S. feltiae*

Table 5 Effect of different VOCs on the chemotactic response of the EPN species, at pure concentration after 24 h

	2-Ethyl-1-hexanol	Bornyl acetate	2,4-Di-tert-butylphenol	Terpinolene	α-Pinene	Control
Sc	−0.11 ± 0.06Aabc	−0.16 ± 0.04Aab	0.05 ± 0.02Cd	−0.23 ± 0.04Aa	−0.11 ± 0.03Ab	−0.02 ± 0.03Ac
Sf	−0.04 ± 0.06ABbc	0.04 ± 0.1Bd	0.02 ± 0.02BCcd	−0.23 ± 0.03Aa	−0.08 ± 0.04Ab	−0.06 ± 0.01Ab
Sk	0.09 ± 0.01Cc	0.13 ± 0.05Cc	−0.02 ± 0.03ABb	−0.05 ± 0.04Bb	−0.14 ± 0.02Aa	−0.07 ± 0.02Ab
Hb	0.01 ± 0.01Bc	0.04 ± 0.04BCc	−0.03 ± 0.02Ab	0 ± 0Cc	−0.1 ± 0.02Aa	−0.01 ± 0.04Abc

Each data point represents the mean CI ± S.E. Each data with the same letter are not significantly different ($p > 0.05$). The capital letters indicate statistically significant differences among different EPN species and same VOC. The small letters indicate statistically significant differences among different VOCs within the same EPN species

Sc, *Steinernema carpocapsae*; *Sf*, *Steinernema feltiae*; *Sk*, *Steinernema kraussei*; *Hb*, *Heterorhabditis bacteriophora*; Ambusher, *S. carpocapsae*; Cruiser, *S. kraussei* and *H. bacteriophora*; Intermediate, *S. feltiae*

(Weissteiner et al. 2012), only nematode species *H. bacteriophora* responded to Terpinolene (as a weak repellent). Terpinolene is a VOC, which is released by undamaged carrot roots (Weissteiner 2010). Our results suggest that EPN are able to distinguish herbivore-induced chemicals from chemicals that are typical for healthy roots.

In our investigation, two distinct VOCs concentrations were used. A pure concentration, which does not reflect a concentration found near plant roots (Köllner et al. 2004), had a bigger influence on IJ movement than a concentration of 0.03 ppm. However, we are aware that such laboratory studies do not reflect a nematode's true behavior in nature because of exposure to different conflicting chemical signals (Bais et al. 2006). Kruitbos et al. (2009) suggested that EPNs may be habitat specialists and highlighted the difficulties of studying soil-transmitted parasites in non-soil media.

Plant roots emit an incredible variety of compounds, which are known to affect interactions between plants (Erb et al. 2013; Hiltpold et al. 2013) and other organisms (Bonkowski et al. 2009). The active role plants play in recruiting natural enemies, like belowground herbivores,

has been recently demonstrated in a few plant species (Rasmann et al. 2012). EPN host-finding is mediated by both long-range cues that facilitate root zone finding, as well as shorter-range cues, that facilitate host localization within the root zone (Hiltpold et al. 2011; Turlings et al. 2012; Demarta et al. 2014). Recently, Hallem et al. (2011) reported positive chemotaxis of the two EPN species *H. bacteriophora* and *S. carpocapsae* to several VOCs such as methyl salicylate, hexanol, heptanol, undecyl acetate, and 4, 5-dimethylthiazole. Interestingly, they showed that several volatiles repelled the nematodes. Dillman et al. (2012) reported that EPN host-seeking behavior is stimulated by a wide range of host-derived odorants. Similar effects of VOCs on the behavior of EPNs were also observed in our investigation. Terpinolene repelled both *Steinernema* and *Heterorhabditis* species in our investigation. Weissteiner and Schütz (2006) reported that Terpinolene is a VOC released from the undamaged roots of cultivated carrots. Our results suggest that EPN are able to distinguish herbivore-induced chemicals from chemicals that are typical for healthy roots. Our findings could support the theory of Ali et al. (2011). Ali et al. (2011) suggest that selection of an herbivore-induced signaling response should be directionally stronger toward channeling resources for production of a distress signal only when necessary because a constant release would likely carry a high physiological cost (Heil 2008; van Dam 2009; Degenhardt et al. 2009; Robert et al. 2013). Our conclusion is also supported by the VOC α-pinene (released from undamaged carrot roots) (Weissteiner and Schütz 2006), which was a weak repellent of *S. carpocapsae* and *S. kraussei*. The other tested VOCs in our investigation (Bornyl acetate, 2, 4-Di-tert-butylphenol, and 2-Ethyl-hexanol) acted inconsistently (as a weak repellents or weak attractants).

Most VOCs that are involved in belowground tritrophic interactions remain unknown but an increasing effort is being made in this field of research. Understanding more of these complex interactions would not only allow a better understanding of the rhizosphere but could also offer ecologically sound alternatives in pest management of agricultural systems (Hiltpold et al. 2010; Turlings et al. 2012).

Author contribution

ŽL: designed and performed the experiments and wrote the manuscript. ST: analyzed the data. All authors read and approved the manuscript.

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