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Root-Emitted Volatile Organic Compounds from *Daucus carota* Modulate Chemotaxis in *Phasmarhabditis* and *Oscheius* Nematodes

Emre Sen [1], Tamás Lakatos [1], Tímea Tóth [2], Stanislav Trdan [3] and Žiga Laznik [3,*]

[1] Research Centre for Fruit Growing, Hungarian University of Agriculture and Life Sciences Horticultural Institute, Elvira Major, 2030 Érd, Hungary; emre.sen@phd.uni-mate.hu (E.S.); tamas.lakatos@gmail.com (T.L.)
[2] Research Institute of Újfehértó, University of Debrecen, Vadastag 2, H-4244 Újfehértó, Hungary; toth.timea@agr.unideb.hu
[3] Department of Agronomy, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia; stanislav.trdan@bf.uni-lj.si
* Correspondence: ziga.laznik@bf.uni-lj.si

Abstract

Root-emitted volatile organic compounds (VOCs) play a critical role in below-ground ecological interactions by mediating communication between plants, pests, and their natural enemies. This study investigates the chemotactic behavior of three slug-parasitic nematode species—*Phasmarhabditis papillosa*, *Oscheius myriophilus*, and *Oscheius onirici*—in response to four carrot (*Daucus carota*) root-derived VOCs: α-pinene, terpinolene, bornyl acetate, and 2-ethyl-1-hexanol. Using a modified Petri dish assay, infective juveniles (IJs) were exposed to each compound across four concentrations (pure, 1000 ppm, 10 ppm, and 0.03 ppm), and their directional movement was quantified using a chemotaxis index (CI). The results revealed strong species-specific and concentration-dependent patterns. *O. myriophilus* exhibited the highest motility and repellency, particularly toward bornyl acetate and terpinolene, indicating its potential for use in VOC-guided biocontrol strategies. *O. onirici* showed moderate but consistent attraction to most VOCs, while *P. papillosa* exhibited generally weak or repellent responses, especially at higher concentrations. None of the compounds tested functioned as strong attractants (CI $\geq$ 0.2), suggesting that plant-derived VOCs alone may not be sufficient to direct nematode recruitment under field conditions. However, their integration with other biotic cues could enhance nematode-based "lure-and-infect" systems for sustainable slug control in carrot cropping systems.

Keywords: carrot; volatile organic compounds (VOCs); *Oscheius myriophilus*; *Phasmarhabditis papillosa*; *Oscheius onirici*; chemotaxis; biocontrol; slug-parasitic nematodes; root volatiles; sustainable agriculture

Academic Editor: John P. Thompson

Received: 24 June 2025
Revised: 24 July 2025
Accepted: 24 July 2025
Published: 25 July 2025

Citation: Sen, E.; Lakatos, T.; Tóth, T.; Trdan, S.; Laznik, Ž. Root-Emitted Volatile Organic Compounds from *Daucus carota* Modulate Chemotaxis in *Phasmarhabditis* and *Oscheius* Nematodes. *Agronomy* **2025**, *15*, 1793. https://doi.org/10.3390/agronomy15081793

1. Introduction

Carrot (*Daucus carota* L.) is among the most widely cultivated root vegetables globally, valued both for fresh consumption and industrial processing. However, its shallow root system renders it especially vulnerable to soil-dwelling pests such as terrestrial slugs (Gastropoda: Stylommatophora). Slug herbivory can devastate young seedlings and cause surface scarring and mucus contamination in mature roots, significantly reducing marketability [1,2]. In some European regions, slug damage is responsible for rendering up to 80% of carrot yields non-commercial [3], with global agricultural losses from slugs estimated at 50–90% [4].

In subterranean ecosystems, volatile organic compounds (VOCs) are key mediators of communication between plant roots and soil organisms [5,6]. Root-emitted volatile compounds (VOCs), particularly in response to herbivore attack, can attract natural enemies such as entomopathogenic nematodes (EPNs) and slug-parasitic nematodes, facilitating indirect plant defense [7,8]. These nematodes locate hosts through chemical cues, invade them, and reproduce internally, ultimately leading to host death and nematode proliferation in the rhizosphere.

The nematode *Phasmarhabditis papillosa* (Schneider) (Rhabditida: Rhabditidae) is an emerging slug-parasitic species. Its infective juveniles (IJs) actively seek out gastropod hosts, penetrate their bodies, and complete a reproductive cycle within the host before emerging to reinfect new hosts [9]. Similarly, *Oscheius myriophilus* (Poinar) and *Oscheius onirici* [10], originally described as entomopathogens, have recently been recognized for their facultative parasitism of slugs [11,12]. *O. myriophilus* exhibits dual reproductive strategies (hermaphroditic and gonochoristic) and demonstrates strong chemotactic responses to slug mucus [13,14], while *O. onirici*, a hermaphroditic generalist, has shown high efficacy against multiple insect species [15].

The limitations of synthetic molluscicides—including short-lived efficacy, environmental risks, and regulatory restrictions—have intensified interest in biological alternatives [16]. Commercial products such as Nemaslug® (a.i. *Phasmarhabditis hermaphrodita* (Schneider) have been applied for slug control for decades, yet concerns remain regarding their cost-effectiveness and host range [17]. Recent studies have highlighted the efficacy of *P. papillosa*, *P. californica*, and *P. hermaphrodita* in controlling slug species such as *Theba pisana* (Müller) at higher inoculum densities, with promising results compared to chemical molluscicides [18]. These nematodes exhibit chemotaxis toward gastropod-derived cues such as mucus and feces [19], indicating their potential as targeted biocontrol agents.

Despite growing knowledge about nematode–host interactions, little is known about how root-emitted VOCs from economically important crops like carrot influence the behavior of slug-parasitic nematodes. Carrot root VOCs, primarily composed of mono- and sesquiterpenes—such as α-pinene, sabinene, terpinolene, β-caryophyllene, and γ-bisabolene—contribute not only to aroma and flavor but also to plant defense [20–22]. These compounds diffuse effectively in soil and are shaped by genetic and environmental factors, making them potent signals in root-associated ecological networks [23–25].

While some volatile organic compounds (VOCs), such as terpinolene, are known to repel EPNs, others—such as α-pinene and 2-ethyl-1-hexanol—have been shown to attract specific species under certain conditions [14,26]. However, the behavioral ecology of slug-parasitic nematodes in response to root-derived VOCs remains largely unexplored. To date, only one study has directly examined this interaction: Laznik et al. [8] investigated the chemotactic responses of *P. papillosa*, *O. myriophilus*, and *O. onirici* to volatiles emitted by *Brassica nigra* L. roots damaged by *Delia radicum* L. larvae. They found that benzonitrile functioned as a strong attractant—particularly for *O. myriophilus* and *O. onirici*—whereas allyl isothiocyanate and phenylethyl isothiocyanate acted as repellents or nematicides. These effects varied with compound concentration and environmental temperature, underscoring the complexity of VOC-mediated signaling in soil ecosystems.

Despite these insights, the impact of VOCs from non-Brassicaceous hosts—such as carrot—on slug-parasitic nematode behavior remains virtually unstudied. This knowledge gap is especially relevant given the distinct volatile profiles of Apiaceae crops and the growing interest in exploiting below-ground chemical cues for sustainable pest control. The current study addresses this gap by evaluating whether herbivore-induced carrot root volatiles can influence the foraging behavior of three slug-parasitic nematode species.

Although *P. papillosa*, *O. myriophilus*, and *O. onirici* are not known to naturally associate with carrot roots, they were selected based on their confirmed parasitism of slugs and proven responsiveness to chemical stimuli [8,11,14]. Rather than documenting an existing ecological association, our goal was to test whether specific carrot-emitted VOCs—especially those linked to herbivory—can act as behavioral cues for slug-parasitic nematodes. Investigating this potential sensitivity is a crucial step toward assessing the feasibility of VOC-guided "lure-and-infect" strategies in integrated pest management.

In this context, we examined the chemotactic responses of infective juveniles from the three nematode species to selected carrot VOCs. By testing across a range of concentrations, we aimed to identify species-specific preferences and behavioral thresholds. Our findings offer mechanistic insights into nematode–VOC interactions and may inform the development of targeted, VOC-enhanced biocontrol approaches for use in carrot cropping systems and other below-ground agroecosystems.

2. Materials and Methods

2.1. Collection, Isolation, and Storage Preparation of Nematodes

In this study, we utilized indigenous strains of *P. papillosa* (GenBank accession no. MT800511.1), *O. myriophilus* (OP684306.1), and *O. onirici* (PQ876382), whose presence in Slovenia was recently verified [8,11].

To maintain these nematodes, in vivo culturing was performed using freeze-killed specimens of the Spanish slug (*Arion vulgaris* Moquin-Tandon) as the host substrate. Following a 10-day incubation period, nematodes were recovered from the decomposed slug tissues through a modified extraction technique tailored for entomopathogenic nematodes (EPNs) [8]. The procedure included centrifugation in a 5% sodium hypochlorite solution, which facilitated the separation of nematodes from residual organic material. Subsequently, the nematode suspension was washed twice with distilled water to remove any remaining contaminants, yielding a purified preparation of infective juveniles (IJs).

The obtained IJs were suspended in an M9 buffer—a physiological saline solution comprising KH_2PO_4, Na_2HPO_4, NaCl, and NH_4Cl—commonly used to maintain nematode viability and osmotic stability [8]. The suspensions were stored at 4 °C until use. Viability was routinely assessed prior to experimental application by analyzing a random sample of approximately 100 IJs under a stereomicroscope. The sample was placed in a droplet of M9 buffer on a microscope slide, and individual nematodes were evaluated for motility. Active nematodes displaying sinusoidal movement or twitching were classified as viable, whereas immobile or degenerated individuals were considered non-viable.

Only batches stored for no longer than 14 days and exhibiting viability rates above 95% were deemed suitable for use in chemotaxis bioassays to ensure a consistent physiological condition and behavioral responsiveness.

2.2. Tested Volatile Compounds

The volatile organic compounds (VOCs) selected for this study were based on the work of Weissteiner et al. [27], who investigated root-emitted VOCs from organically grown carrot plants in response to herbivory by *Melolontha hippocastani* Fabricius larvae (cockchafer grubs). Using gas chromatography–mass spectrometry (GC–MS), their study identified distinct volatile profiles associated with undamaged versus herbivore-damaged carrot roots. Specifically, undamaged roots primarily released the terpenoids α-pinene and terpinolene, while herbivore-damaged roots emitted increased levels of bornyl acetate and 2-ethyl-1-hexanol—indicating herbivory-induced shifts in root volatile emissions.

To explore the potential behavioral relevance of these compounds, synthetic standards of the four VOCs were procured from Sigma-Aldrich (Merck, St. Louis, MO, USA) with

the following purities: α-pinene (≥98%), terpinolene (≥90%), bornyl acetate (≥98%), and 2-ethyl-1-hexanol (≥99%). Each compound was tested at four concentration levels: undiluted (pure), 1000 ppm, 10 ppm, and 0.03 ppm. This gradient was designed to simulate a broad spectrum of exposure scenarios, ranging from high concentrations potentially found near damaged root zones to low concentrations more typical of VOC diffusion through bulk soil. While pure compounds exceed natural levels, their inclusion follows standard practice in nematode chemotaxis research (e.g., [8,28]) and serves to identify upper behavioral thresholds, including potential repellency or overstimulation effects.

All VOCs were diluted in 96% ethanol, selected for its established use in agar-based nematode assays due to its miscibility with water and chemical stability. Although concerns have been raised about solvent effects, our design included solvent-only controls (10 µL ethanol applied on both treatment and control sites) to rule out behavioral artifacts. Across all nematode species, chemotaxis index values for these controls remained near zero, confirming that ethanol at this concentration and volume did not influence nematode movement. Alternative solvents such as n-hexane were considered but deemed less suitable due to their volatility, poor agar compatibility, and increased handling risk.

Stock solutions were freshly prepared for each experiment by serial dilution using precision micropipettes, followed by vortexing to ensure homogeneity. All assays were conducted within a climate-controlled chamber to minimize compound degradation and maintain consistent environmental conditions (22 °C, 75% RH, complete darkness). This setup allowed for a controlled and repeatable assessment of VOC-induced chemotaxis under laboratory conditions that, while simplified, provide ecologically meaningful insights into species-specific nematode behavior.

2.3. Chemotaxis Assay

Chemotaxis assays (see Figure 1) were conducted following the method originally described by O'Halloran and Burnell [28], with modifications from Laznik et al. [8,12,26]. All assays were performed using 9 cm diameter plastic Petri dishes (polystyrene, sterile, Greiner Bio-One), each filled with 25 mL of 1.6% technical agar (Biolife, Milan, Italy). The agar was prepared in a chemotaxis buffer composed of 5 mM potassium phosphate (pH 6.0), 1 mM $CaCl_2$, and 1 mM $MgSO_4$, creating conditions that promote nematode motility and chemotactic response.

Each Petri dish was divided into three areas (see Figure 1): a treated area, a control area, and a central area. Volatile organic compounds (VOCs) were applied to the left side of each dish (the treated area), and an equal volume of solvent (96% ethanol) was applied to the right side (the control area). Approximately 100 infective juveniles (IJs) were suspended in the M9 buffer and pipetted into the center of each dish (the central area), ensuring consistent initial positioning.

The M9 buffer, used to suspend IJs for placement, was prepared as follows: 6.0 g Na_2HPO_4 (final concentration: 42.5 mM), 3.0 g KH_2PO_4 (22.0 mM), and 0.5 g NaCl (8.5 mM) were dissolved in 1 L of distilled water. After autoclaving, 1.0 mL of sterile 1 M $MgSO_4 \cdot 7H_2O$ was added (final concentration: 1.0 mM). The buffer was stored at 4 °C and used throughout the assays to maintain osmotic stability and nematode viability.

Each treatment was replicated ten times, and the full assay series was repeated independently three times to ensure statistical validity. To prevent VOC cross-contamination, only one volatile was tested per assay series, and each Petri dish was individually sealed with Parafilm™ to restrict volatile diffusion within the dish.

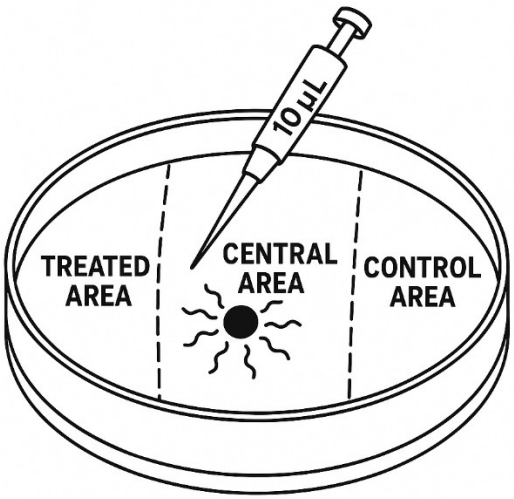

Figure 1. Experimental setup for chemotaxis assays. Each 9 cm polystyrene Petri dish was marked with three reference points (1 cm in diameter) on the underside: one at the center (central area) and two positioned 1.5 cm from the dish edge on the left and right sides (treated and control areas, respectively). For VOC treatments, 10 µL of the test volatile organic compound (VOC) was applied to the left-side reference point (treated area), and 10 µL of 96% ethanol was applied to the right-side reference point (control area) as a solvent control. Subsequently, 100 infective juveniles (IJs) suspended in 10 µL of M9 buffer were placed at the central point. For control treatments, both the treated and control zones received 10 µL of 96% ethanol to eliminate VOC-specific chemotactic cues. This standardized spatial configuration and volume ensured consistency across all replicates, enabling reliable comparison of nematode responses to VOC exposure.

Assays were incubated in a climate-controlled chamber (RK-900 CH, Kambič Laboratory Equipment, Semič, Slovenia) at 22 °C, 75% relative humidity, and in complete darkness to mimic subterranean environments and eliminate light-induced behavioral variation.

After 24 h, dishes were briefly frozen at −20 °C for 3 min to immobilize the nematodes and preserve their final distribution. Nematode positions were analyzed using a Nikon SMZ800N (Nikon Corporation, Tokyo, Japan) stereomicroscope fitted with a 4K UHD HDMI camera (XCAM4K8MPB) at 25× magnification.

Behavioral responses were quantified using the chemotaxis index (CI), calculated as

$$CI = (\%\ \text{of IJs in the treated area} - \%\ \text{of IJs in the control area})/100\%$$

The CI thus provides a standardized measure of nematode behavioral response, quantitatively capturing the degree of attraction or repulsion elicited by each tested volatile compound.

The CI values ranged from 1.0 (indicating complete attraction) to −1.0 (indicating complete repulsion). Based on the calculated CI, compounds were classified as follows: attractants ($CI \geq 0.2$), weak attractants ($0.2 > CI \geq 0.1$), neutral ($-0.1 \leq CI < 0.1$), weak repellents ($-0.2 \leq CI < -0.1$), and repellents ($CI \leq -0.2$) [8,12,26].

2.4. Statistical Analysis

In the chemotaxis assay, the directional movement of nematodes from the central area to the treated and control area of the Petri dish—interpreted as a preferential behavioral response—was analyzed using a paired Student's *t*-test. Statistical significance was determined at $p < 0.05$.

To assess differences in nematode behavior across treatments, the proportion of infective juveniles (IJs) that migrated to the outer zones or remained within the central zone was calculated for each replicate. These data were analyzed using a two-way analysis

of variance (ANOVA), with significance set at $p < 0.05$. The analysis included the main effects of VOC identity, nematode species, and VOC concentration, as well as their interactions. Among all tested combinations, only the three-way interaction between nematode species, VOC, and VOC concentration yielded statistically significant and biologically meaningful results.

In addition, a separate two-way ANOVA was performed on the chemotaxis index (CI) values to compare the overall responsiveness of different nematode species to the tested VOCs. Where significant effects were detected, mean comparisons were conducted using Duncan's multiple range test ($p < 0.05$) to identify differences between treatment groups.

All data are presented as mean ± standard error (SE). Statistical analyses were conducted using Statgraphics Plus for Windows, Version 4.0 (Statistical Graphics Corp., Manugistics, Inc., Rockville, MD, USA), and visualizations were created using Microsoft Excel 2010.

3. Results

3.1. Nematode Motility

In this study, motility was quantified as the proportion of infective juveniles (IJs) that migrated from the central area of the Petri dish toward either the treatment or control zones (Figure 1). This directional movement served as a behavioral proxy for evaluating the effects of nematode species, volatile organic compound (VOC) identity, concentration, and their interactions on locomotor activity.

The analysis of variance (ANOVA) revealed that nematode species identity had the strongest overall influence on IJ motility, followed by VOC type and concentration (Table 1). Several significant interaction effects were also observed—particularly between species and VOC, and the three-way interaction involving species, VOC, and concentration—highlighting the complex and context-dependent nature of nematode chemosensory behavior. In contrast, neither temporal nor spatial replication effects were significant, confirming the reproducibility and robustness of the experimental design across independent trials.

Table 1. ANOVA results for the directional movement of infective juveniles (IJs) from the central area toward treatment and control areas in the Petri dish.

Factor	Sum of Squares	Df	F	*p*
Nematode species (S)	6553.79	2	16.50	<0.0001
VOCs (V)	5187.43	4	6.53	<0.0001
VOCs concentration (Vc)	3193.48	4	4.02	0.0032
Temporal replication	2949.21	9	1.65	0.0987
Spatial replication	421.03	2	1.06	0.3455
S × V	13,600.11	8	8.56	<0.0001
S × Vc	3320.59	8	2.09	0.0350
V × Vc	6672.95	16	2.10	0.0156
S × V × Vc	11,820.66	32	1.86	0.0081
Residual	104,860.66	528		
Total (corrected)	150,484.60	599		

Mean motility patterns also differed significantly among species. *O. myriophilus* exhibited the highest baseline motility (~18%), followed by *O. onirici* (~12.5%) and *P. papillosa* (~9.5%) (Figure 2). These interspecific differences reflect inherent variation in activity levels and responsiveness to environmental cues, even under control conditions.

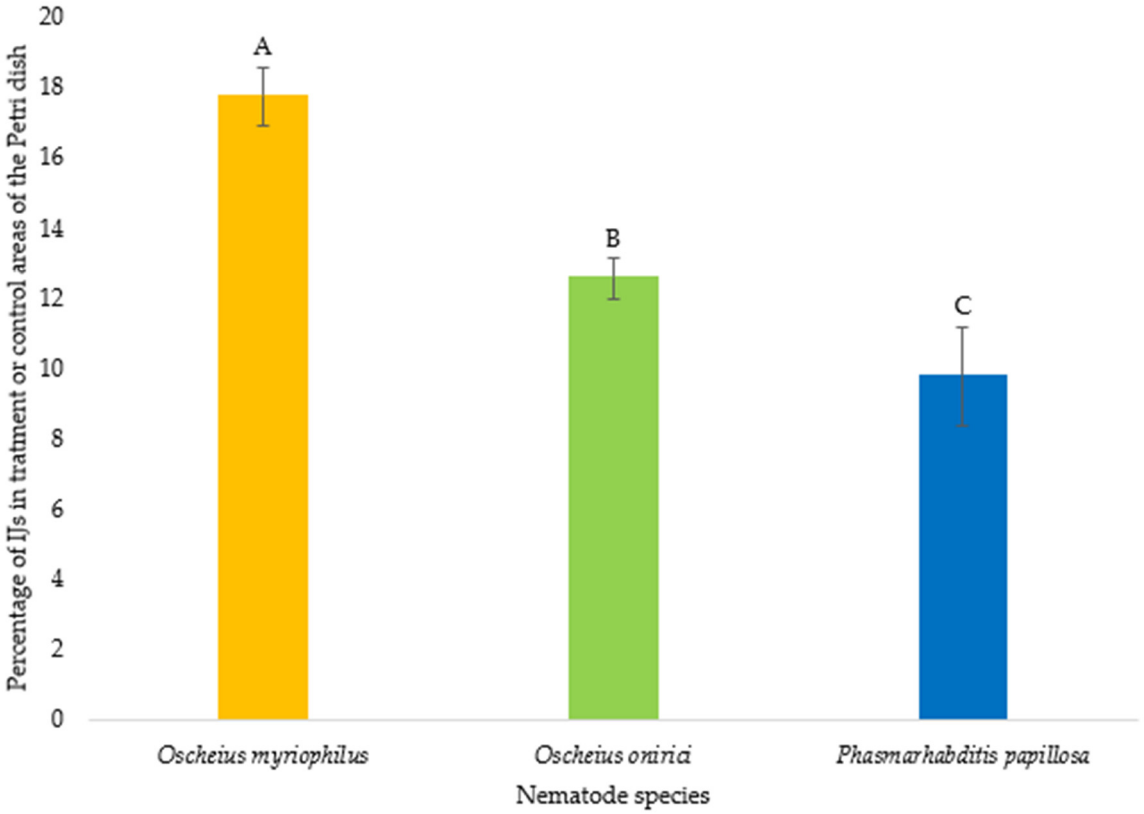

Figure 2. Mean percentage (±standard error) of infective juveniles (IJs) of three nematode species—*Oscheius myriophilus*, *Oscheius onirici*, and *Phasmarhabditis papillosa*—that migrated from the central area to the treatment or control areas of the chemotaxis assay dish. Different capital letters above the bars indicate statistically significant differences among species based on Duncan's multiple range test ($p < 0.05$).

Together, these findings underscore the importance of species-specific traits, chemical identity, and concentration in modulating nematode movement, while validating the experimental system as a reliable tool for assessing VOC-driven behavior in soil nematodes.

Figure 2 shows the mean percentage (±SE) of infective juveniles (IJs) migrating from the central area to the treatment or control areas of the Petri dish for the three nematode species. *O. myriophilus* exhibited the highest motility (~18%), followed by *O. onirici* (~12.5%), while *P. papillosa* showed the lowest response (~9.5%). These results indicate significant interspecific differences in baseline locomotor activity under control conditions.

Figure 3 illustrates the chemotactic responses of the three nematode species to 2-ethyl-1-hexanol at four concentrations (pure, 1000 ppm, 10 ppm, and 0.03 ppm) and a 96% ethanol control. Across all concentrations, *O. myriophilus* and *P. papillosa* consistently exhibited higher motility than *O. onirici*. The strongest response was observed for *O. myriophilus* at 1000 ppm (~27%), closely followed by *P. papillosa* (~25%). *O. onirici* responded weakly, with motility values ranging from ~11 to 13%. Within species, both *O. myriophilus* and *P. papillosa* displayed a clear dose-dependent pattern, peaking at 1000 ppm and decreasing with dilution. In contrast, *O. onirici* maintained low and relatively uniform responsiveness, suggesting reduced sensitivity to this compound.

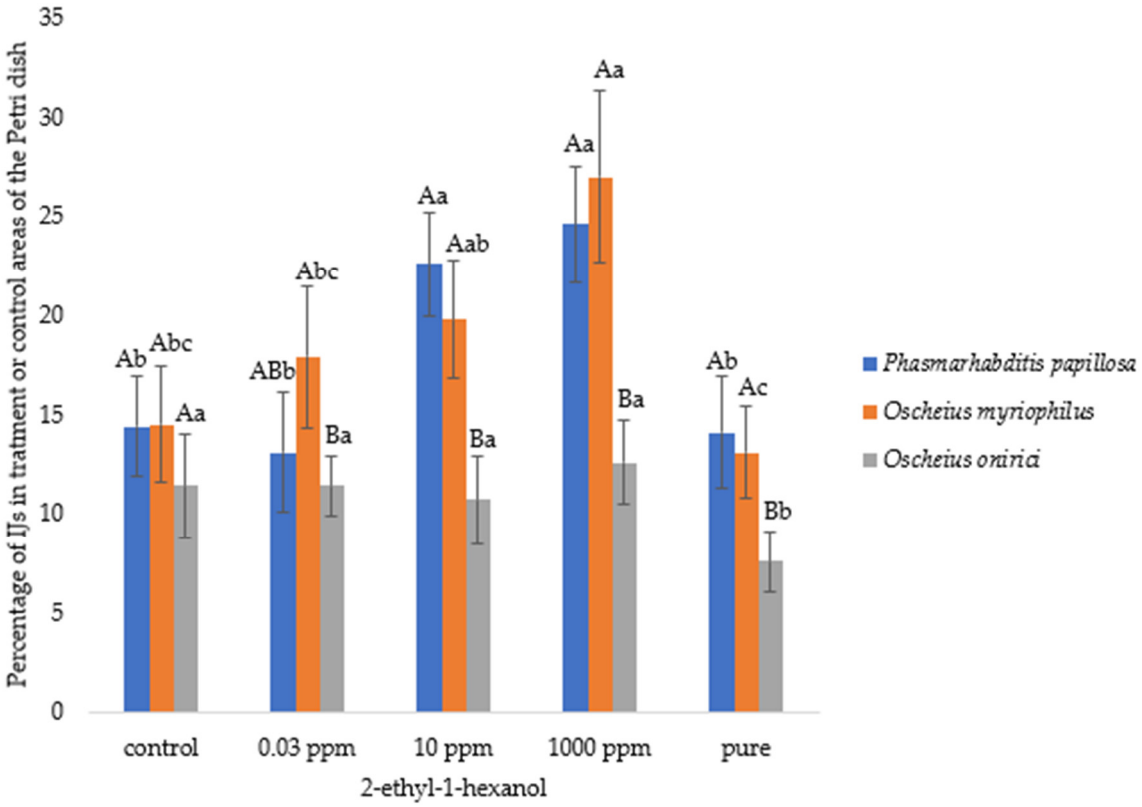

Figure 3. Chemotactic responses of three slug-parasitic nematode species to 2-ethyl-1-hexanol.

Mean percentage (±standard error) of infective juveniles (IJs) of *Phasmarhabditis papillosa*, *Oscheius myriophilus*, and *Oscheius onirici* migrating from the central zone of a Petri dish toward either the treatment (2-ethyl-1-hexanol at four concentrations: pure, 1000 ppm, 10 ppm, 0.03 ppm) or the solvent control (96% ethanol). IJ motility (%) was calculated as the proportion of individuals moving from the central application point toward either side of the dish (treatment or control), expressed as a percentage of the total IJs per replicate. Different capital letters above the bars denote statistically significant differences among nematode species within the same VOC concentration, while lowercase letters denote significant differences within the same nematode species across VOC concentrations (Duncan's multiple range test, $p < 0.05$).

Figure 4 presents responses to α-pinene across the same range of concentrations. *O. myriophilus* again showed the highest motility, with peak activity at 1000 ppm (~22%). *O. onirici* responded moderately, particularly at lower concentrations (10 ppm and 0.03 ppm), with motility stabilizing around 13–14%. *P. papillosa* exhibited low responsiveness throughout (<10%), except in the control (~14%), suggesting little attraction to α-pinene. These results highlight species-specific differences, with *O. myriophilus* exhibiting a strong dose-dependent response, while *O. onirici* and *P. papillosa* were less responsive.

Mean percentage (±standard error) of infective juveniles (IJs) of *Phasmarhabditis papillosa*, *Oscheius myriophilus*, and *Oscheius onirici* migrating from the central zone of the Petri dish toward either α-pinene (tested at pure, 1000 ppm, 10 ppm, and 0.03 ppm) or the 96% ethanol control. IJ motility (%) was calculated as the proportion of IJs leaving the central application point and moving toward either the treatment or control area, expressed as a percentage of the total nematodes per replicate. Different capital letters above the bars indicate statistically significant differences among nematode species within the same VOC concentration, while lowercase letters denote significant differences within the same species across different concentrations (Duncan's multiple range test, $p < 0.05$).

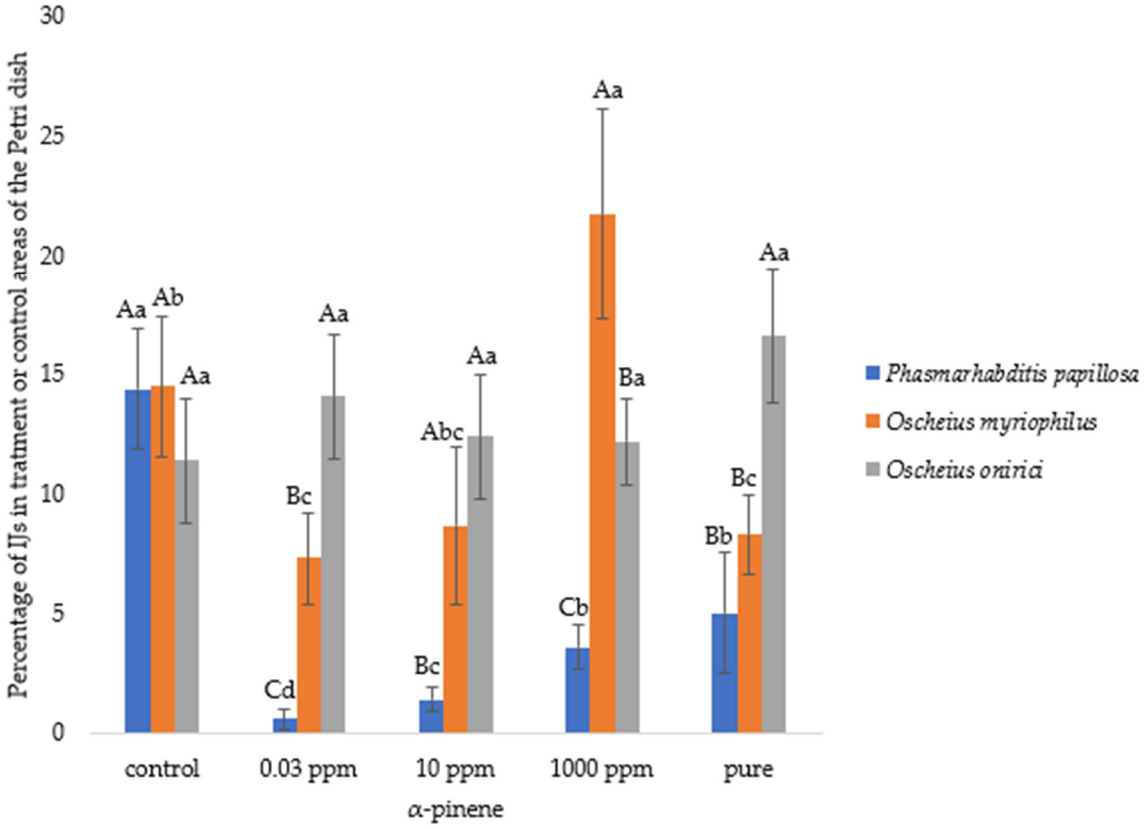

Figure 4. Chemotactic responses of three slug-parasitic nematode species to α-pinene.

Figure 5 displays nematode responses to bornyl acetate. *O. myriophilus* again demonstrated the highest motility, with a peak at 0.03 ppm (~28%) and sustained attraction at higher concentrations. *O. onirici* showed intermediate responses (~12–18%), and *P. papillosa* remained consistently unresponsive, with values below 7% across all treatments. Within species, *O. myriophilus* responded robustly across concentrations, suggesting high sensitivity, particularly to low doses. *O. onirici* showed a slight decline with dilution, while *P. papillosa* was minimally affected by treatment.

Mean percentage (±standard error) of infective juveniles (IJs) of *Phasmarhabditis papillosa*, *Oscheius myriophilus*, and *Oscheius onirici* migrating from the central zone of the Petri dish toward bornyl acetate (tested at pure, 1000 ppm, 10 ppm, and 0.03 ppm) or the 96% ethanol control. IJ motility (%) was calculated as the proportion of individuals leaving the central application point and moving toward either the treatment or control area, expressed as a percentage of the total nematodes per replicate. Different capital letters above the bars indicate statistically significant differences among nematode species within the same VOC concentration, while lowercase letters indicate significant differences within the same nematode species across concentrations (Duncan's multiple range test, $p < 0.05$).

Figure 6 summarizes the responses to terpinolene. *O. myriophilus* again exhibited the highest attraction, peaking at 0.03 ppm (~27%), followed closely by 10 ppm and 1000 ppm (~24–26%). *O. onirici* responded moderately (~13–19%) across concentrations, with a slight decrease at intermediate doses. *P. papillosa* showed low responsiveness (~4–10%) but increased activity in the control condition (~14%), suggesting limited attraction to terpinolene itself.

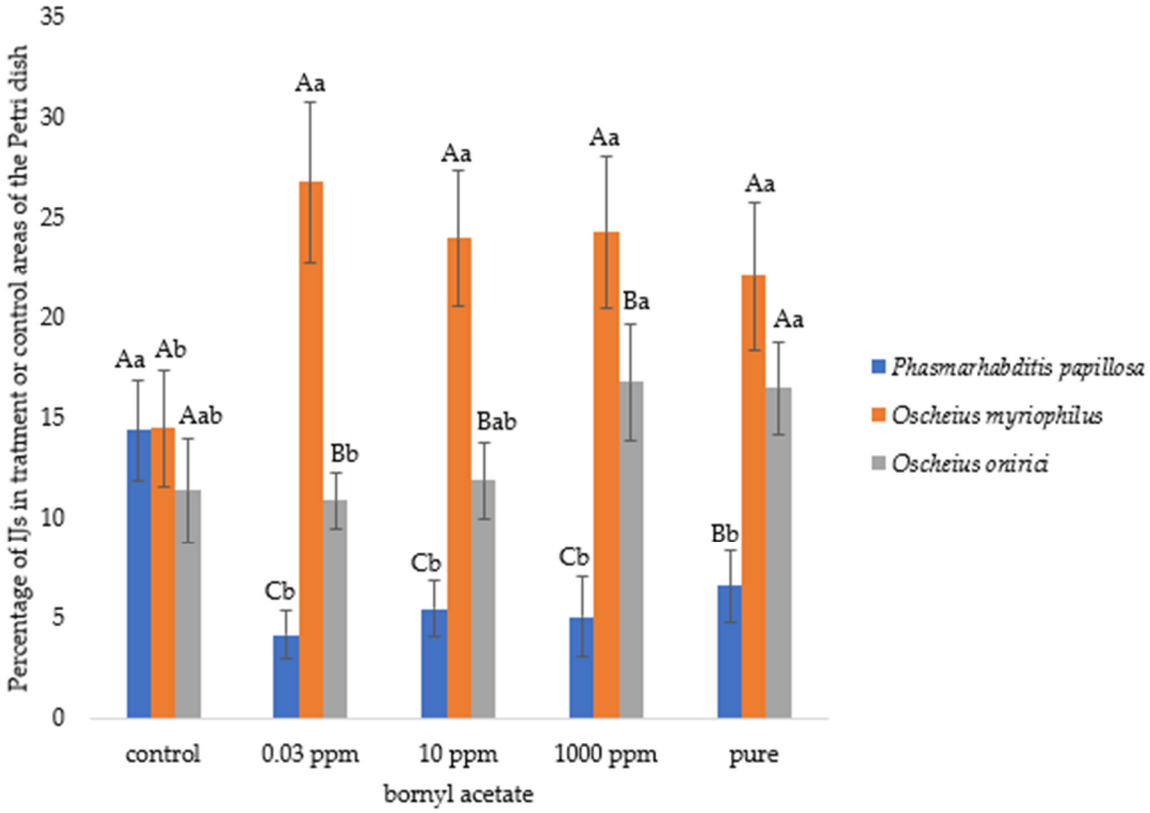

Figure 5. Chemotactic responses of three slug-parasitic nematode species to bornyl acetate.

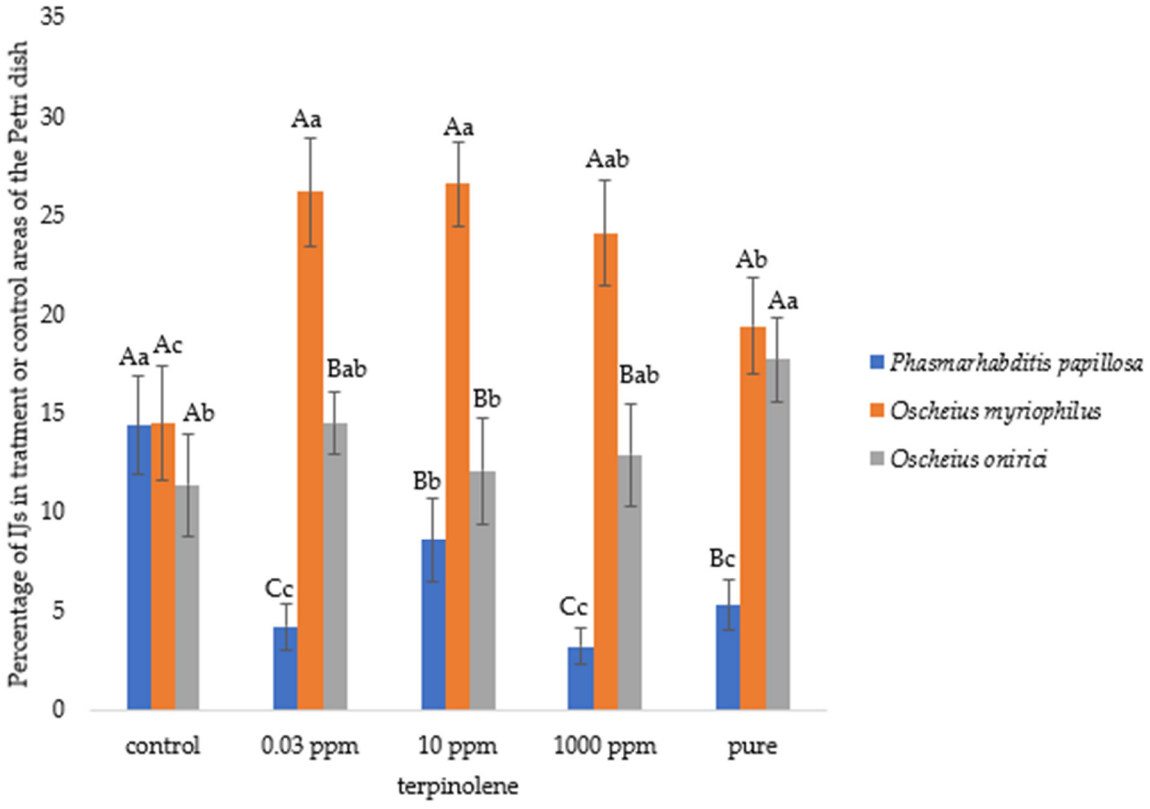

Figure 6. Chemotactic responses of three slug-parasitic nematode species to terpinolene.

Regarding the mean percentage (±standard error) of infective juveniles (IJs) of *Phasmarhabditis papillosa*, *Oscheius myriophilus*, and *Oscheius onirici* migrating from the central zone of the Petri dish toward terpinolene (tested at pure, 1000 ppm, 10 ppm, and

0.03 ppm) or the 96% ethanol control, IJ motility (%) was calculated as the proportion of nematodes leaving the central application point and moving toward either the treatment or control area, expressed as a percentage of the total IJs per replicate. Different capital letters above the bars indicate statistically significant differences among nematode species within the same VOC concentration, while lowercase letters indicate significant differences within the same nematode species across concentrations (Duncan's multiple range test, $p < 0.05$).

Overall, the chemotaxis assays revealed pronounced species-specific and dose-dependent variation in nematode responses to the tested VOCs. *O. myriophilus* consistently demonstrated the highest motility and sensitivity across all compounds and concentrations, particularly at intermediate or low doses. *O. onirici* showed moderate and relatively stable responses, while *P. papillosa* displayed low and uniform responsiveness. These trends mirrored baseline motility patterns observed in the control assays and highlight clear interspecific differences in chemosensory behavior. The findings suggest that *O. myriophilus* may be the most responsive species for potential applications involving VOC-guided biological control.

3.2. Chemotaxis Index

Directional movement in response to volatile organic compounds (VOCs) was further quantified using the chemotaxis index (CI), providing a standardized measure of attraction or repulsion across treatments. ANOVA results (Table 2) confirmed that nematode species, VOC identity, and VOC concentration all significantly influenced chemotactic behavior. The strongest effects were observed for species identity, followed by compound concentration and chemical structure. Notably, a significant three-way interaction among species, VOC, and concentration was also detected, reinforcing the context-dependent nature of nematode responses.

Table 2. ANOVA results for the chemotaxis index values.

Factor	Sum of Squares	Df	F	*p*
Nematode species (S)	0.44	2	26.24	<0.0001
VOCs (V)	0.08	4	2.51	0.0410
VOCs concentration (Vc)	0.16	4	4.72	0.0010
Temporal replication	0.10	9	1.37	0.1970
Spatial replication	0.01	2	0.65	0.5211
S × V	0.12	8	1.74	0.0315
S × Vc	0.16	8	2.42	0.0140
V × Vc	0.51	16	3.82	<0.0001
S × V × Vc	1.35	32	5.09	<0.0001
Residual	4.39	528		
Total (corrected)	7.32	599		

As in the motility assay, temporal and spatial replication had no significant effects on CI values, confirming the consistency of the experimental design.

Among the three species, *O. onirici* exhibited the highest and most consistently positive CI values across several treatments—particularly in response to bornyl acetate and terpinolene—indicating a general tendency toward weak attraction. *O. myriophilus*, despite showing the highest motility, often displayed neutral or negative CI values at higher concentrations of α-pinene and bornyl acetate, suggesting possible overstimulation or repellent effects at elevated doses. In contrast, *P. papillosa* showed largely neutral or weakly negative responses across all VOCs, with no clear evidence of attraction.

These results indicate that chemotactic behavior is shaped not only by species identity but also by specific chemical profiles and dose-dependent sensitivity. The CI-based patterns

complement the motility data, revealing that high activity levels do not always correspond to strong directional preference, and that VOC perception varies substantially among nematode taxa.

The chemotaxis index (CI) values for three nematode species in response to varying concentrations of four volatile organic compounds (VOCs) are summarized in Table 3.

Table 3. Chemotaxis index (CI ± standard error) of three nematode species—*P. papillosa* (PP), *O. myriophilus* (OM), and *O. onirici* (OO)—in response to varying concentrations of four volatile organic compounds (VOCs): 2-ethyl-1-hexanol, α-pinene, bornyl acetate, and terpinolene, including a 96% ethanol control. CI values are interpreted as follows: values ≥ 0.2 indicate an attractant effect; values between 0.1 and 0.2 indicate a weak attractant; values between −0.1 and 0.1 indicate no effect; values between −0.1 and −0.2 indicate a weak repellent; and values ≤ −0.2 are considered a repellent. Different capital letters within columns indicate statistically significant differences ($p < 0.05$) among nematode species for the same VOC and concentration, while different lowercase letters within rows indicate significant differences ($p < 0.05$) among concentrations for the same nematode species, based on Duncan's multiple range test.

			2-ethyl-1-hexanol		
	Pure	1000 ppm	10 ppm	0.03 ppm	Control
PP	−0.10 ± 0.02 Aab	−0.11 ± 0.01 Aa	−0.05 ± 0.02 Ac	−0.07 ± 0.02 Abc	0.02 ± 0.01 Ad
OM	−0.01 ± 0.02 Bbc	−0.08 ± 0.04 Aa	−0.03 ± 0.01 Aab	−0.06 ± 0.02 Aa	0.01 ± 0.01 Ac
OO	0.04 ± 0.01 Cb	0.00 ± 0.02 Ba	0.02 ± 0.02 Bab	0.01 ± 0.01 Ba	0.00 ± 0.01 Aa
			α-pinene		
	Pure	1000 ppm	10 ppm	0.03 ppm	Control
PP	0.02 ± 0.01 Bb	−0.01 ± 0.01 Ba	−0.01 ± 0.01 Aa	0.00 ± 0.00 Aa	0.02 ± 0.01 Ab
OM	−0.05 ± 0.02 Ab	−0.20 ± 0.04 Aa	−0.03 ± 0.01 Ab	0.03 ± 0.01 Bc	0.01 ± 0.01 Ac
OO	−0.03 ± 0.01 Aa	−0.01 ± 0.01 Bab	0.04 ± 0.01 Bc	−0.02 ± 0.02 Aab	0.00 ± 0.01 Ab
			bornyl acetate		
	Pure	1000 ppm	10 ppm	0.03 ppm	Control
PP	−0.02 ± 0.01 Ba	−0.02 ± 0.01 Ba	−0.01 ± 0.01 Ba	−0.01 ± 0.02 Bab	0.02 ± 0.01 Ab
OM	−0.15 ± 0.03 Aab	−0.20 ± 0.03 Aa	−0.13 ± 0.02 Ab	−0.19 ± 0.03 Aa	0.01 ± 0.01 Ac
OO	0.04 ± 0.02 Cb	0.01 ± 0.03 Bab	0.05 ± 0.02 Cb	0.02 ± 0.02 Bab	0.00 ± 0.01 Aa
			Terpinolene		
	Pure	1000 ppm	10 ppm	0.03 ppm	Control
PP	0.00 ± 0.00 Bb	0.00 ± 0.00 Bb	−0.05 ± 0.02 Aa	0.00 ± 0.00 Bb	0.02 ± 0.01 Ac
OM	−0.03 ± 0.02 Ac	−0.10 ± 0.04 Aab	−0.08 ± 0.03 Abc	−0.14 ± 0.02 Aa	0.01 ± 0.01 Ad
OO	0.05 ± 0.03 Cbc	0.03 ± 0.02 Cb	0.05 ± 0.03 Bbc	0.09 ± 0.02 Cc	0.00 ± 0.01 Aa

Across all VOCs, *O. onirici* consistently exhibited the highest CI values, particularly in response to bornyl acetate and terpinolene, where it reached up to 0.09, classifying it as no effect to weak attractant depending on the concentration. Notably, *O. onirici* showed positive CI values at all concentrations of bornyl acetate and terpinolene, unlike the other two species. In contrast, *O. myriophilus* generally displayed negative CI values, especially in response to α-pinene (−0.20 at 1000 ppm) and bornyl acetate (−0.20 at 1000 ppm), indicating a weak repellent effect at higher concentrations. Its responses to terpinolene also suggested repellency, with CI values ranging from −0.03 to −0.14.

P. papillosa exhibited weak to moderate repellency in response to 2-ethyl-1-hexanol, particularly at pure and 1000 ppm concentrations (CI = −0.10 and −0.11), while responses to other compounds generally fell within the "no effect" range. Its CI values in control treatments ranged from 0.00 to 0.02 across all compounds, confirming that the solvent alone did not elicit a chemotactic response.

Across all treatments, none of the VOCs triggered strong attractant behavior (CI $\geq$ 0.2) in any species. Instead, *O. onirici* showed the most consistent neutral-to-slightly-attractive responses, while *O. myriophilus* and *P. papillosa* were more variable and frequently repelled, especially at higher VOC concentrations. These results highlight clear species-specific and concentration-dependent differences in nematode chemotaxis, with *O. onirici* showing the greatest potential for positive VOC-guided movement and *O. myriophilus* the most pronounced avoidance behavior under specific conditions.

4. Discussion

This study demonstrates that volatile organic compounds (VOCs) emitted by carrot roots can influence the chemotactic behavior of slug-parasitic nematodes in a species-specific and concentration-dependent manner. These findings contribute to our understanding of below-ground multitrophic interactions and reinforce the ecological relevance of root-emitted VOCs in shaping nematode foraging behavior [5–7].

Among the three tested nematode species, *O. myriophilus* exhibited the highest motility and responsiveness across VOC treatments. Its sensitivity to low concentrations of bornyl acetate and terpinolene aligns with prior work demonstrating strong chemotaxis toward slug mucus and other host-derived cues [13,14]. This suggests *O. myriophilus* may utilize plant-emitted volatiles in combination with biotic cues during host location. The ability to respond to ecologically realistic concentrations (e.g., 0.03–10 ppm) further supports its potential use in VOC-enhanced biocontrol strategies [29–31].

In contrast, *P. papillosa* showed low motility and weak or negative chemotactic responses to most VOCs. This subdued profile may reflect a narrower chemosensory range, alternative host-searching strategies, or sensitivity to VOC-induced overstimulation, particularly at higher doses of α-pinene and 2-ethyl-1-hexanol [19]. As a recently characterized species [32,33], further research is needed to clarify the behavioral ecology of *P. papillosa* and its reliance on contact versus airborne cues.

O. onirici displayed moderate but stable responses, especially to bornyl acetate and terpinolene, with generally positive CI values across concentrations. Its consistent yet less intense chemotaxis suggests a generalist orientation strategy, which is in line with its broad host range and previously documented parasitism of both insects and slugs [8,10,15]. In fluctuating or chemically complex soil environments, such behavioral consistency may prove advantageous for biological control deployment.

The compounds tested in this study reflect herbivore-induced changes in carrot root emissions [27], and their chemotactic impact varied both by identity and concentration. Bornyl acetate and terpinolene elicited the most consistent attraction across species, particularly at low to intermediate concentrations—consistent with their known role in root defense and signaling [34,35]. α-Pinene and 2-ethyl-1-hexanol, by contrast, triggered more variable or repellent responses, especially at higher doses, supporting previous findings that certain VOCs can function as deterrents or toxicants to nematodes [8,26].

These findings underscore the critical role of concentration thresholds in mediating nematode chemotaxis—a concept well established in entomopathogenic nematode research [7,36]. The observed three-way interaction among species, VOC identity, and concentration reflects the intricate interplay between chemosensory adaptation and compound-specific signaling in guiding foraging behavior.

Although our four-dose gradient (pure, 1000 ppm, 10 ppm, and 0.03 ppm) effectively captured species- and compound-specific patterns across a broad behavioral window, incorporating additional intermediate concentrations (e.g., 700, 500, 300, 100, and 50 ppm) would enhance the resolution of dose–response relationships. Such finer-scale testing could reveal non-linear or bell-shaped response curves, as commonly observed in nematode

chemotaxis assays [37]. Pinpointing inflection thresholds for attraction or repulsion is essential for translating laboratory results to field conditions, where VOC dispersion and stability are influenced by environmental heterogeneity.

We acknowledge this methodological limitation and now emphasize the importance of expanded concentration gradients in future research to refine behavioral thresholds and identify minimal effective doses. This information will be pivotal for optimizing VOC-based "lure-and-infect" strategies under field-relevant scenarios. Comparable nuanced response patterns have been observed in systems involving *Heterorhabditis* and *Steinernema* spp., further validating the ecological and practical relevance of such chemotactic variability [28,37,38].

While this study utilized simplified Petri dish assays to isolate and quantify the chemotactic effects of individual carrot-emitted VOCs, such systems remain a widely accepted first step in nematode behavioral studies [29,38]. However, we acknowledge that agar-based platforms represent artificial conditions that differ markedly from real soil environments. Critical soil factors such as moisture gradients, microbial metabolism, particle structure, and VOC sorption–desorption dynamics can significantly influence volatile perception and nematode movement [31,39].

Notably, nematodes have been shown to exhibit divergent behavioral responses to the same compound when tested on agar versus soil substrates. For example, El-Borai et al. [40] demonstrated that *Steinernema riobrave* displayed attraction to certain VOCs in agar-based assays but avoided them in sand-based olfactometers. Such context-dependent behavior highlights the importance of substrate selection in chemotaxis research and underscores the need for complementary assays in more ecologically realistic systems.

In light of this, future work should expand beyond agar assays and incorporate soil olfactometer trials or semi-field conditions to evaluate nematode responsiveness under natural substrate constraints. Numerous studies have successfully implemented such methods to assess VOC-mediated nematode orientation and validate behavioral patterns observed in vitro [7,35,38,41]. Integrating these approaches will be crucial for assessing the consistency, reliability, and field applicability of "lure-and-infect" strategies informed by VOC-guided behavior.

Additionally, although the slug-parasitic nematodes tested in this study are not currently known to associate with carrot roots under natural conditions, our aim was not to confirm such ecological links. Instead, we sought to explore whether carrot-emitted VOCs—particularly those upregulated during herbivory—could serve as candidate orientation cues. Our findings confirm that VOCs can indeed modulate nematode behavior in species- and concentration-specific ways, supporting the broader utility of plant volatiles in designing targeted biocontrol strategies.

5. Conclusions

This study demonstrates that root-emitted VOCs, particularly bornyl acetate and terpinolene, can influence the foraging behavior of slug-parasitic nematodes in a species- and concentration-dependent manner. The differential chemotactic responses observed across *P. papillosa*, *O. myriophilus*, and *O. onirici* reveal distinct olfactory sensitivities and highlight *O. myriophilus* as a promising candidate for VOC-guided biocontrol applications.

Although tested in a simplified assay system, the findings provide a mechanistic foundation for integrating plant-derived volatiles into multi-cue "lure-and-infect" strategies. Future research should focus on expanding VOC concentration gradients, validating nematode responses in soil environments, and assessing potential synergies with biotic cues. Such efforts will be critical for translating VOC-mediated nematode orientation into robust, field-ready solutions for sustainable slug management.

Author Contributions: Ž.L., S.T., T.T. and T.L. designed the project and conducted the chemotactic experiment; E.S. conducted the chemotactic assay; E.S. and Ž.L. analyzed the data and wrote the paper. All authors have read and agreed to the published version of the manuscript.

Funding: This work was conducted within project J4-50135 and research program P4-0431, both funded by the Slovenian Research Agency. Part of this research was also funded through the Professional Tasks from the Field of Plant Protection program, supported by the Ministry of Agriculture, Forestry, and Food of the Republic of Slovenia–Phytosanitary Administration. Additional support was provided by the Pannónia Scholarship Program, which contributed to the research mobility and academic collaboration aspects of this study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: Special thanks are given to Jaka Rupnik and Karolina Kralj for their technical assistance.

Conflicts of Interest: The authors declare no conflicts of interest.

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