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Responses of Parasitic Nematodes to Volatile Organic Compounds Emitted by *Brassica nigra* Roots

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Abstract: Parasitic nematodes, particularly those in the Rhabditidae family, are vital components of belowground ecosystems, contributing to pest regulation and sustainable agriculture. This study investigated the chemotactic responses of three nematode species—*Phasmarhabditis papillosa*, *Oscheius myriophilus*, and *O. onirici*—to volatile organic compounds (VOCs) emitted by *Brassica nigra* roots under herbivory by *Delia radicum* larvae. Using a chemotaxis assay, the effects of five VOCs (dimethyl sulfide, dimethyl disulfide, allyl isothiocyanate, phenylethyl isothiocyanate, and benzonitrile) were tested at two concentrations (pure and 0.03 ppm) and two temperatures (18 °C and 22 °C). The results revealed that VOCs and temperature significantly influenced nematode responses, while nematode species and VOC concentration showed limited effects. Benzonitrile consistently demonstrated strong chemoattractant properties, particularly for *O. myriophilus* and *O. onirici*. Conversely, allyl isothiocyanate exhibited potent nematicidal effects, inhibiting motility and causing mortality. Dimethyl disulfide and dimethyl sulfide elicited moderate to strong attractant responses, with species- and temperature-dependent variations. Significant interactions between VOCs, temperature, and nematode species highlighted the complexity of these ecological interactions. These findings emphasize the ecological roles of VOCs in mediating nematode behavior and their potential applications in sustainable pest management. Benzonitrile emerged as a promising candidate for nematode-based biocontrol strategies, while allyl isothiocyanate showed potential as a direct nematicidal agent. The study underscores the importance of integrating chemical cues into pest management systems to enhance agricultural sustainability and reduce reliance on chemical pesticides.

Keywords: parasitic nematodes; *Phasmarhabditis papillosa*; *Oscheius myriophilus*; *Oscheius onirici*; chemotaxis; volatile organic compounds; benzonitrile; phenylethyl isothiocyanate; belowground interactions

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1. Introduction

Parasitic nematodes play a vital role in belowground ecosystems, where they contribute to pest regulation and serve as natural enemies of herbivores [1–3]. Among them, species from the family Rhabditidae, such as *Oscheius myriophilus* (Poinar), are gaining attention for their potential in biological pest control. Additionally, entomopathogenic nematodes (EPNs) belonging to the Heterorhabditidae and Steinernematidae families are

widely recognized for their effectiveness in managing insect pests [4]. Representative species, including *Steinernema feltiae* Filipjev, *Steinernema carpocapsae* [Weiser], *Heterorhabditis bacteriophora* Poinar, and *Phasmarhabditis hermaphrodita* (Schneider), have demonstrated a strong potential as biocontrol agents, providing sustainable alternatives to chemical pesticides [4,5]. Furthermore, recent studies have highlighted the efficacy of *Phasmarhabditis papillosa* (Schneider) Andrassy in targeting mollusk pests, particularly slugs, which pose a significant threat to agricultural productivity [3,6,7]. The integration of these nematodes into pest management strategies not only offers environmentally friendly pest control solutions but also supports biodiversity and promotes ecological balance.

Nematodes utilize chemical cues, including carbon dioxide (CO_2) and volatile organic compounds (VOCs) emitted by plants and associated organisms, to locate hosts or prey [8–10]. Plant secondary metabolites, particularly those produced by members of the Brassicaceae family, play a dual role in plant defense and ecological signaling [11–16]. Crucifers are renowned for their production of glucosinolates and their breakdown products, such as isothiocyanates, nitriles, and thiocyanates [13]. These compounds not only defend plants against herbivores and pathogens but also mediate interactions with soil-dwelling organisms, including nematodes [15,16].

Plants exhibit remarkable specificity in their responses to herbivory, fine-tuning VOC emissions to correspond with the particular herbivore species attacking them [12,17,18]. This chemical signaling can deter herbivores and recruit natural enemies like nematodes as part of an indirect defense strategy [10,11]. For example, VOCs such as (E)-β-caryophyllene and 4,5-dimethylthiazole released by insect-damaged roots attract EPNs, whereas others, such as hexanal and terpinolene, act as repellents [11,14,19]. Nematodes are also sensitive to electrical fields and chemical gradients emitted by host organisms and plant roots, further illustrating their reliance on complex environmental cues for host location [20].

The cabbage root fly (*Delia radicum* [L.]), a natural root herbivore of wild and cultivated *Brassica* species, induces the release of VOCs, including sulfur-containing compounds and glucosinolate breakdown products, from the roots of infested plants such as *Brassica nigra* L. [13]. These emissions provide an opportunity to study the chemotactic responses of parasitic nematodes to VOCs as part of plant–nematode–herbivore interactions.

The primary objective of this research is to enhance our understanding of the chemotactic behavior of parasitic nematodes, specifically *P. papillosa*, *O. myriophilus*, and *O. onirici*, a recently described member of the *Oscheius* genus, in response to volatile organic compounds (VOCs) emitted by *B. nigra* roots following damage by *D. radicum* larvae. While the behavioral responses of entomopathogenic nematodes (EPNs) have been well documented in previous studies [11,12,15], the chemotactic behaviors of *P. papillosa*, *O. myriophilus*, and *O. onirici* remain largely unexplored, necessitating further investigation. The first objective of this study is to determine whether chemotaxis varies based on species and temperature. Temperature is a critical factor influencing nematode activity and host-seeking behavior, and understanding species-specific responses under different thermal conditions will provide insights into their ecological adaptability and potential efficacy in varying climatic environments. The second objective is to assess the behavioral effects of VOCs emitted by insect-damaged *B. nigra* roots on selected nematode species. Previous research has shown that herbivore-induced VOCs can attract natural enemies, including nematodes, as part of an indirect plant defense strategy [10,11,13]. However, the role of specific sulfur-containing VOCs in guiding parasitic nematodes has not been thoroughly investigated, particularly in the context of *D. radicum* infestation. Examining nematode responses to these compounds will help elucidate their role in belowground multitrophic interactions. The third objective is to explore whether these VOCs contribute to indirect plant defense mechanisms. Plants are known to release specific chemical signals in response to herbivory,

attracting beneficial organisms that aid in pest suppression [12,17,18]. Identifying whether VOCs from *B. nigra* roots serve as attractants for parasitic nematodes could provide novel insights into plant–nematode–herbivore interactions and offer potential applications in biological control strategies. By addressing these objectives, this study aims to advance our understanding of belowground chemical signaling and its ecological significance while contributing to the development of sustainable pest management approaches.

2. Materials and Methods

2.1. Nematode Collection, Isolation and Preparation for Storage

The Spanish slug (*Arion vulgaris* Moquin-Tandon) specimens were collected from trial fields at the Biotechnical Faculty in Ljubljana (46°04′ N, 14°31′ E, 299 m a.s.l.) during June and September 2023. Species identification was conducted using standardized identification charts [21]. A total of 200 slugs were collected and rinsed with 0.9% saline solution, following the protocol outlined by Pieterse et al. [6]. Each slug was dissected individually while alive, and nematodes recovered from their body cavities were preserved in 80% ethanol for molecular analysis [7].

The nematodes were subsequently cultured in the laboratory (in vivo) using freeze-killed *A. vulgaris* slugs as a substrate. After 10 days, nematode cultures were washed according to a protocol for entomopathogenic nematodes (EPNs) [1,2]. This involved centrifuging the nematodes in a 5% sodium hypochlorite solution, followed by two additional cycles of cleaning with distilled water to obtain infective juveniles (IJs) from the suspension. The IJs were stored in an M9 buffer at 4 °C, and only those less than two weeks old with a viability of over 95% were utilized.

Native populations of *P. papillosa* (GenBank accession number MT800511.1) and *O. myriophilus* (GenBank accession number OP684306.1) were also subcultured for this study, as their presence was recently confirmed in Slovenia [3,7].

2.2. Molecular Identification

For identification of nematode strains, amplification of internal transcribed spacer (ITS), and small subunit rRNA gene (SSU) was performed. Genomic DNA was extracted from single nematode individuals. An infective juvenile was placed in a 0.5 mL micro centrifuge tube containing 10 µL of worm lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45 Tween 20, 0.01% gelatin and 60 µg/mL proteinase K). The tube was frozen at −80 °C for 10 min and then incubated at 65 °C for 1 h, followed by 10 min at 95 °C to inactivate the proteinase K. The lysates were cooled on ice and centrifuged at 12,000× *g* for 2 min, and 2.5 µL of the supernatant was used in the PCR reaction as a template. PCR was carried out using primer TW81 and AB28 for amplification of ITS region in accordance with Hominick et al. [22], and 18S-F and 18S-R for amplification of the SSU region in accordance with Liu et al. [23]. The PCR products were separated by electrophoresis on 1.5% (*w/v*) agarose gels in 1 x TBE buffer and were purified using QIAquick PCR and Gel Clean Up kit (Qiagen, Hilden, Germany). Sanger sequencing was performed by Microsynth (Balgach, Switzerland). The overlapping partial sequences were aligned by ClustalX 2.1 and trimmed manually. Sample DNA sequences were compared with the database of the National Centre for Biotechnology Information (NCBI) using the BLAST search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 2 February 2025)).

2.3. Tested Volatile Compounds

The selection of volatile organic compounds (VOCs) for this study was based on the findings of Crespo et al. [13], who identified VOC emissions from the roots of *B. nigra* plants under attack by cabbage root fly larvae (*D. radicum*). Using proton transfer

reaction mass spectrometry (PTR-MS) and gas chromatography mass spectrometry (GC-MS), they identified five key VOCs released by damaged *B. nigra* roots: (1) dimethyl sulfide (DMS), (2) dimethyl disulfide (DMDS), (3) allyl isothiocyanate (AITC), (4) phenylethyl isothiocyanate (PEITC), and (5) benzonitrile (BN).

For our investigation, we used synthetically produced versions of these compounds (Sigma-Aldrich, now part of Merck). We purchased the following VOCs with the indicated purities: allyl isothiocyanate (95%), dimethyl sulfide (99%), dimethyl disulfide (98%), phenylethyl isothiocyanate (98%), and benzonitrile (99%). These compounds were tested both at their pure concentrations (as provided by the supplier) and at a diluted concentration of 0.03 ppm, which corresponds to the average VOC concentration found in the soil 10 cm from the root system of *B. nigra* [24].

To achieve the desired 0.03 ppm concentration, pure VOCs were diluted in 96% ethanol. The specific dilution process for each compound involved carefully measuring the appropriate volume of the pure VOC stock and diluting it with ethanol to achieve the target concentration. Each dilution was thoroughly mixed using a centrifuge to ensure uniformity before applying the resulting suspension in laboratory bioassays. This approach allowed for precise and consistent VOC concentrations throughout the experiments, ensuring the accuracy of the testing conditions.

2.4. Chemotaxis Assay

The chemotaxis assay (see Figure 1), adapted from O'Halloran and Burnell [25] and further refined by Laznik and Trdan [14], was performed using Petri dishes (ø = 9 cm) filled with 25 mL of 1.6% technical agar (Biolife, Milan, Italy) supplemented with 5 mM potassium phosphate buffer (pH 6.0), 1 mM $CaCl_2$, and 1 mM $MgSO_4$. Each treatment included 10 replicates, and all experiments were repeated three times to ensure reliability.

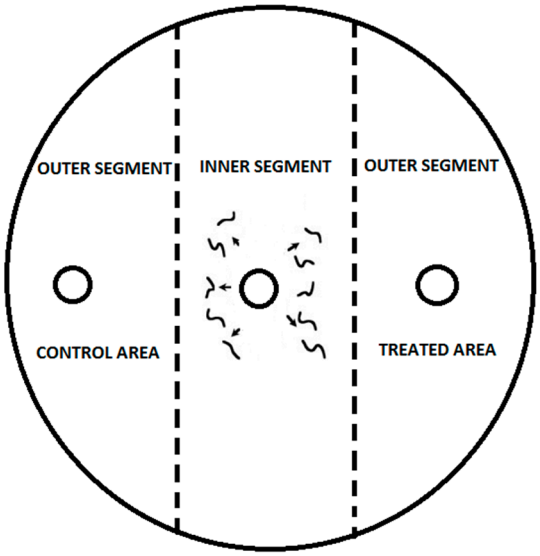

Figure 1. To prepare the experimental setup, three 1 cm diameter marks were drawn on the bottom of each Petri dish: one at the center and two positioned 1.5 cm from the edge on the right and left sides. The volatile organic compound (VOC) under investigation, at a selected concentration, was applied to the right side of the agar (treated area), while the left side was treated with 10 µL of 96% ethanol as the control (control area). VOCs were applied immediately before introducing 100 infective juveniles (IJs) of nematodes, delivered in a 50 µL droplet at the center of the agar. For control setups, 96% ethanol was applied to both sides, and 100 IJs in a 50 µL droplet were placed at the center.

To avoid cross-contamination of VOCs between the treatments, only one VOC was tested at a time in each set of Petri dishes. The Petri dishes were securely covered with

laboratory film (Parafilm) to minimize evaporation and prevent VOCs from escaping or spreading to adjacent dishes. This setup ensured that the VOCs remained confined to the designated treatment area, maintaining the integrity of the experiment.

The Petri dishes were incubated in a rearing chamber (RK-900 CH, Kambič Laboratory Equipment, Semič, Slovenia) under dark conditions at 18 and 22 °C with 75% relative humidity. Nematodes were allowed to move freely for 24 h. After this period, the dishes were frozen at −20 °C for 3 min to immobilize the nematodes. Nematode counts in the treatment and control areas were then conducted using a Nikon SMZ800N binocular microscope at 25× magnification.

The chemotaxis index (CI) was calculated using the formula adapted from Bargmann and Horvitz [26] and Laznik and Trdan [14]:

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

The CI values ranged from 1.0 (indicating perfect attraction) to −1.0 (indicating perfect repulsion). Based on the CI, compounds were categorized as attractants ($CI \geq 0.2$), weak attractants ($0.2 > CI \geq 0.1$), neutral ($-0.1 \leq CI < 0.1$), weak repellents ($-0.2 \leq CI < -0.1$), or repellents ($CI \leq -0.2$) [14].

2.5. Statistical Analysis

In the chemotaxis assay, a paired Student's *t*-test was used to evaluate the directional movement of nematodes from the inner to the outer segments of the Petri dish, indicating a preferential response. Differences between treatments were considered significant at $p < 0.05$. To assess response levels among different species, the average percentage of infective juveniles (IJs) moving to the outer segments or remaining in the inner segments was calculated for each dish. These data were analyzed using two-way analysis of variance (ANOVA) with a significance threshold of $p < 0.05$. The significance of individual factors (VOCs, nematode species, temperature, VOC concentration, and replications) and their interactions were also tested. Among the ANOVA results, only the interaction between VOCs, nematode species, temperature, and concentration was found to be significant and interpretable.

Additionally, two-way ANOVA was performed on the chemotaxis index (CI) values to compare responses among different nematode species exposed to the tested VOCs. Duncan's multiple range test ($p < 0.05$) was used to separate the means. Data are presented as the mean ± standard error (SE). Statistical analyses were conducted using Statgraphics Plus for Windows 4.0 (Statistical Graphics Corp., Manugistics, Inc., Rockville, MD, USA), and figures were created using MS Office Excel 2010.

3. Results

3.1. Molecular Identification

The ITS and SSU DNA sequences of the newly isolated nematode strain had the highest, 99.76% and 99.60%, similarity to those (LN613265 and LN613261, respectively) obtained from the population used to describe *Oscheius onirici* [27]. The sequences obtained from the Slovenian *O. onirici* strain were deposited in GenBank under accession numbers PQ876382 (ITS) and PQ857195 (SSU).

3.2. Nematode Chemoattraction Towards VOCs

Nematode Motility

In this study, "motility" refers to the movement of nematode infective juveniles (IJs) from the central region to the outer areas of the assay dish. This behavior was measured by observing the distribution of nematodes between the inner (central) and outer zones

of the Petri dish, with the outer zones representing the area where the VOCs were either present (treatment) or absent (control). The percentage of nematodes that migrated to the outer zones was used as an indicator of their motility, which was significantly influenced by multiple factors (Table 1).

Table 1. ANOVA results for the directional movement of infective juveniles (IJs) from the inner to the outer segments of the Petri dish.

Factor	Sum of Squares	Df	F	*p*
Nematode species (S)	1056.2	2	0.58	0.5584
VOCs (V)	23,659.5	5	5.23	0.0001
VOCs concentration (C)	56.3	1	0.06	0.8031
Temperature (T)	13,266.3	1	14.65	0.0001
Temporal replication	9090.7	9	1.12	0.3492
Spatial replication	2240.0	2	1.24	0.2451
S × V	2992.8	10	0.33	0.9729
S × C	2445.0	2	1.35	0.2599
S × T	1402.2	2	0.77	0.4615
V × C	14,199.4	5	3.14	0.0083
V × T	10,561.0	5	2.33	0.0409
S × V × C	16,593.1	10	1.83	0.0520
S × V × T	17,130.6	10	1.89	0.0434
Residual	583,594.6	662		
Total (Corrected)	707,145.0	719		

Among the primary factors, VOCs (F = 5.23, $p = 0.0001$) and temperature (F = 14.65, $p = 0.0001$) emerged as highly significant, highlighting their critical roles in driving nematode responses. In contrast, nematode species, VOC concentration, and replication effects did not yield significant results, indicating uniform responses across these variables. Noteworthy interactions included VOCs × temperature (V × T, F = 2.33, $p = 0.0409$) and VOCs × concentration (V × C, F = 3.14, $p = 0.0083$), demonstrating that VOC effects are modulated by temperature and concentration levels. Furthermore, the three-way interaction (S × V × T, F = 1.89, $p = 0.0434$) underscored the complex interplay among species, VOCs, and temperature. These findings confirm the pivotal roles of VOCs and temperature in shaping nematode behavior, with the observed interactions providing deeper insight into the nuanced environmental and chemical influences on motility.

At 18 °C among the VOCs tested, benzonitrile (BN) demonstrated the strongest stimulatory effect on IJ motility. Across all nematode species (*P. papillosa*, *O. myriophilus*, and *O. onirici*), BN as a pure compound induced the highest percentage of IJs moving to the outer areas, with *O. myriophilus* and *O. onirici* showing particularly strong responses (Figure 2). Conversely, allyl isothiocyanate (AITC) exhibited a potent nematicidal effect at pure concentrations, significantly reducing motility in all nematode species. Minimal movement was observed, highlighting the toxic nature of AITC at this concentration. Notably, all IJs of *P. papillosa*, *O. myriophilus*, and *O. onirici* died during the experiment when exposed to pure AITC (Figure 2). Dimethyl disulfide (DMDS) and dimethyl sulfide (DMS) elicited moderate to high motility responses, with *O. myriophilus* and *O. onirici* showing stronger responses at pure concentrations compared to the 0.03 ppm treatment (Figure 2). Control treatments (96% ethanol) resulted in consistently minimal motility across all nematode species, confirming the absence of any stimulatory or inhibitory effects in the control setup. These findings at 18 °C underscore the diverse impacts of VOCs on nematode motility, with benzonitrile acting as a potent chemoattractant, strongly stimulating movement, while allyl isothiocyanate displayed significant toxicity at higher concentrations, leading to a marked reduction in nematode motility and survival.

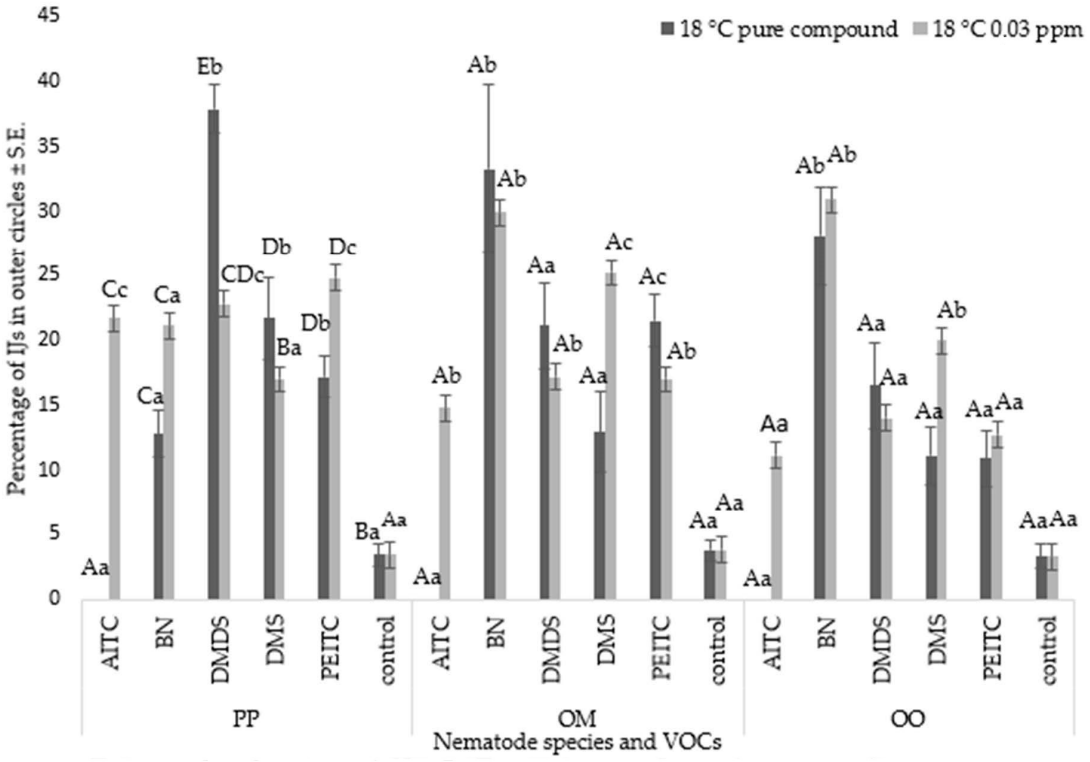

Figure 2. The chart illustrates the percentage of infective juveniles (IJs) of different nematode species present in the outer segments after 24 h at 18 °C, as influenced by the VOC type, VOC concentration, and nematode species used in the experiment. Error bars indicate the standard error of the mean. Capital letters denote statistically significant differences ($p < 0.05$) among various VOCs at the same concentration within a single nematode species. Lowercase letters indicate statistically significant differences ($p < 0.05$) among the different nematode species for the same VOC and VOC concentration. The nematode species tested in the experiment include: PP = *Phasmarhabditis papillosa*, OM = *Oscheius myriophilus*, and OO = *Oscheius onirici*. The VOCs used in the experiment are as follows: DMS = dimethyl sulfide, DMDS = dimethyl disulfide, AITC = allyl isothiocyanate, PEITC = phenylethyl isothiocyanate, BN = benzonitrile, and control = 96% ethanol.

At a higher temperature (22 °C), BN continued to induce the highest motility among all tested nematode species, particularly in *P. papillosa* and *O. onirici*. A higher percentage of IJs moved to the outer areas in response to pure BN compared to the 0.03 ppm treatment (Figure 3). DMDS and DMS stimulated moderate motility in all nematode species, with slightly stronger responses observed at pure concentrations compared to the diluted treatment (Figure 3). Again, AITC exhibited a potent nematicidal effect at pure concentrations, significantly reducing motility in all nematode species (Figure 3). The control treatments at 22 °C consistently showed low motility across all nematode species, indicating no stimulatory or inhibitory effects.

These results highlight that temperature influences the chemotactic response of nematodes to VOCs. While benzonitrile consistently acted as the strongest chemoattractant across temperatures, other VOCs, such as AITC, demonstrated limited or toxic effects, with responses varying slightly depending on temperature and concentration.

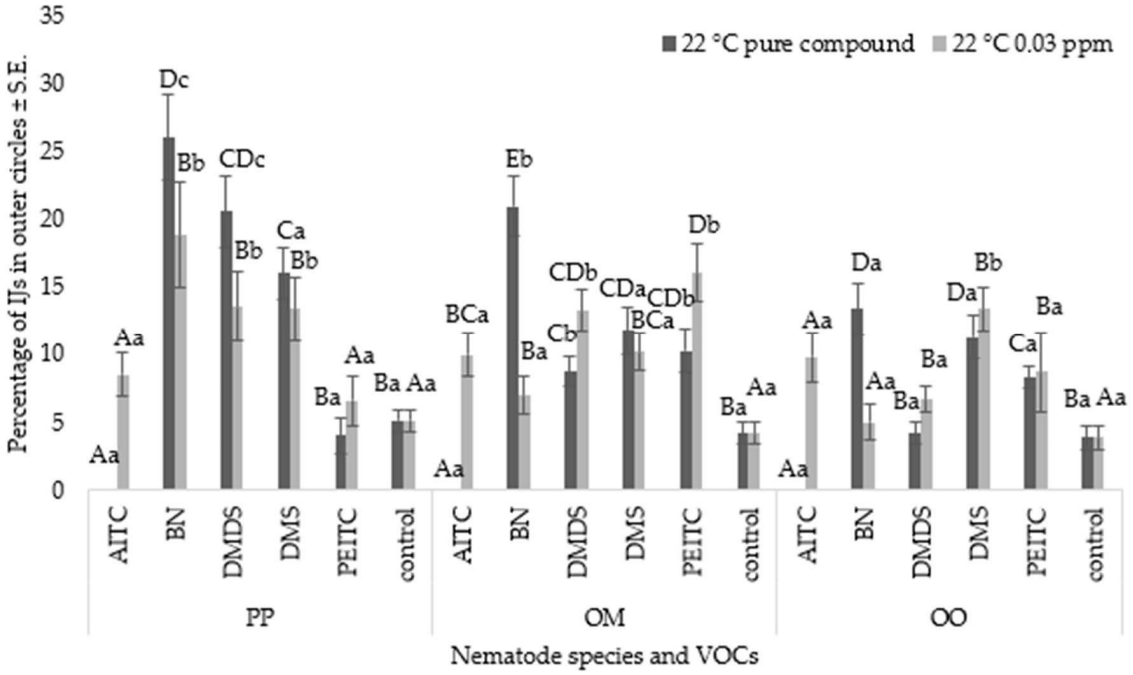

Figure 3. The chart illustrates the percentage of infective juveniles (IJs) of different nematode species present in the outer segments after 24 h at 22 °C, as influenced by the VOC type, VOC concentration, and nematode species used in the experiment. Error bars indicate the standard error of the mean. Capital letters denote statistically significant differences ($p < 0.05$) among various VOCs at the same concentration within a single nematode species. Lowercase letters indicate statistically significant differences ($p < 0.05$) among the different nematode species for the same VOC and VOC concentration. The nematode species tested in the experiment include PP = *Phasmarhabditis papillosa*, OM = *Oscheius myriophilus*, and OO = *Oscheius onirici*. The VOCs used in the experiment are as follows: DMS = dimethyl sulfide, DMDS = dimethyl disulfide, AITC = allyl isothiocyanate, PEITC = phenylethyl isothiocyanate, BN = benzonitrile, and control = 96% ethanol.

3.3. Chemotaxis Index

The movement preference of nematode IJs was assessed using the chemotaxis index (CI). Table 2 presents the results of an ANOVA analysis for the CI values, examining the effects of nematode species, VOCs, VOC concentration, temperature, and replication, as well as their interactions. The main factors influencing the chemotaxis index included VOC type and temperature, both of which showed highly significant effects (F = 29.84, $p = 0.0001$ for VOCs; F = 15.52, $p = 0.0001$ for temperature). Conversely, VOC concentration, temporal replication, and spatial replication did not significantly affect chemotaxis ($p > 0.05$). Among the interactions, the nematode species by VOC interaction (S × V, F = 6.52, $p = 0.0001$) and the VOC by temperature interaction (V × T, F = 16.50, $p = 0.0001$) were significant, indicating that nematode responses varied depending on the specific VOCs and environmental conditions. Furthermore, higher-order interactions such as species by VOC by concentration (S × V × C, F = 2.12, $p = 0.0214$) and species by VOC by temperature (S × V × T, F = 8.71, $p = 0.0001$) were also significant, reflecting the complex interplay between nematode species, chemical cues, and environmental factors. Residual variance accounted for a substantial portion of the total variance, suggesting the presence of other unexplored factors influencing nematode chemotaxis.

The chemotaxis index (CI) values for the nematode species, *P. papillosa*, *O. myriophilus*, and *O. onirici*, under the influence of different VOCs at 18 °C, are summarized in Table 3. Among the pure VOCs, BN demonstrated the strongest chemoattractant properties for *O. myriophilus* and *O. onirici*, with CI values of 0.22 ± 0.05 and 0.23 ± 0.03, respectively.

Conversely, BN had no significant effect on *P. papillosa*, showing a CI value of -0.02 ± 0.01. For DMS and DMDS, the responses varied among the species. DMS elicited a weak attractant response in *P. papillosa* (0.11 ± 0.00) and *O. myriophilus* (0.04 ± 0.02), while DMDS had a weak repellent effect on *O. myriophilus* (-0.11 ± 0.03). PEITC displayed moderate attractant properties for *O. myriophilus* (0.17 ± 0.03) but had a minimal effect on *P. papillosa* and *O. onirici*. AITC and the control treatment (96% ethanol) consistently exhibited no chemoattractant or repellent activity across all species, with CI values of 0.00 ± 0.00.

Table 2. ANOVA results for the chemotaxis index values.

Factor	Sum of Squares	Df	F	*p*
Nematode species (S)	0.0083	2	0.55	0.5774
VOCs (V)	1.1279	5	29.84	0.0001
VOCs concentration (C)	0.0009	1	0.12	0.7263
Temperature (T)	0.1173	1	15.52	0.0001
Temporal replication	0.0786	9	1.16	0.3214
Spatial replication	0.0165	2	1.08	0.4312
S × V	0.4928	10	6.52	0.0001
S × C	0.0088	2	0.58	0.5592
S × T	0.0153	2	1.01	0.3644
V × C	0.0386	5	1.02	0.4049
V × T	0.6238	5	16.50	0.0001
S × V × C	0.1600	10	2.12	0.0214
S × V × T	0.6584	10	8.71	0.0001
Residual	6.4948	682		
Total (Corrected)	8.6428	719		

Table 3. Effect of different volatile organic compounds on the chemotactic response of the nematode species after 24 h at 18 °C. Data represent the mean value of the chemotaxis index ± standard error. Capital letters denote statistically significant differences ($p < 0.05$) among various VOCs at the same concentration within a single nematode species. Lowercase letters indicate statistically significant differences ($p < 0.05$) among the different nematode species for the same VOC and VOC concentration. The VOCs used in the experiment are as follows: DMS = dimethyl sulfide, DMDS = dimethyl disulfide, AITC = allyl isothiocyanate, PEITC = phenylethyl isothiocyanate, BN = benzonitrile, and control = 96% ethanol. The chemotaxis index values are categorized as follows: values ≥ 0.2 are considered as an attractant, values between 0.2 and 0.1 are classified as a weak attractant, values between 0.1 to -0.1 have no effect, values between -0.1 to -0.2 are categorized as a weak repellent, and values ≤ -0.2 are considered as a repellent to nematodes.

VOCs	18 °C		
	Pure Compound		
	Phasmarhabditis papillosa	***Oscheius myriophilus***	***Oscheius onirici***
AITC	0.00 ± 0.00 Ba	0.00 ± 0.00 Ba	0.00 ± 0.00 Aa
BN	−0.02 ± 0.01 Aa	0.22 ± 0.05 Db	0.23 ± 0.03 Db
DMDS	0.05 ± 0.01 Cb	−0.11 ± 0.03 Aa	0.02 ± 0.01 Bb
DMS	0.11 ± 0.00 Db	0.04 ± 0.02 Ca	0.03 ± 0.01 Aa
PEITC	0.01 ± 0.01 Ba	0.17 ± 0.03 Dc	0.06 ± 0.01 Cb
Control	0.00 ± 0.00 Ba	0.00 ± 0.00 Ba	0.00 ± 0.00 Aa

Table 3. *Cont.*

VOCs	18 °C		
	Pure Compound		
	Phasmarhabditis papillosa	***Oscheius myriophilus***	***Oscheius onirici***
	0.03 ppm		
AITC	−0.05 ± 0.03 Aa	0.00 ± 0.04 BCa	−0.04 ± 0.01 Aa
BN	0.12 ± 0.03 CDa	0.22± 0.04 Eb	0.27 ± 0.04 Db
DMDS	0.16 ± 0.01 Dc	−0.08 ± 0.02 Aa	0.04 ± 0.04 BCb
DMS	−0.06 ± 0.03 Aa	0.08 ± 0.02 Db	−0.07 ± 0.03 Aa
PEITC	0.11 ± 0.05 Ca	0.07 ± 0.04 Ca	0.08 ± 0.02 Ca
Control	0.00 ± 0.00 Ba	0.00 ± 0.00 Ba	0.00 ± 0.00 Ba

At the lower VOC concentration (0.03 ppm), BN remained a strong attractant for *O. myriophilus* and *O. onirici*, with CI values of 0.22 ± 0.04 and 0.27 ± 0.04, respectively. For *P. papillosa*, DMDS demonstrated the strongest attractant effect at 0.03 ppm (0.16 ± 0.01). Interestingly, AITC and DMS elicited weak or negligible responses across all species at this concentration. PEITC showed weak attractant activity across all nematode species (Table 3).

The chemotaxis index (CI) values for the nematode species, *P. papillosa*, *O. myriophilus*, and *O. onirici*, under the influence of different VOCs at 22 °C, are summarized in Table 4. At pure concentrations, DMDS elicited the strongest attractant response in *P. papillosa* (CI = 0.13 ± 0.03) and *O. myriophilus* (CI = 0.03 ± 0.02), while *O. onirici* showed negligible movement (CI = 0.02 ± 0.01). DMS had a mild attractant effect on *O. onirici* (CI = 0.07 ± 0.01) but was repellent to *P. papillosa* (CI = −0.06 ± 0.03). BN was a weak attractant for *P. papillosa* (CI = 0.10 ± 0.02) and *O. myriophilus* (CI = 0.08 ± 0.03), while *O. onirici* exhibited no response (CI = 0.00 ± 0.01). Control treatments (96% ethanol) consistently showed no effect on chemotaxis across all species (Table 4).

Table 4. Effect of different volatile organic compounds on the chemotactic response of the nematode species after 24 h at 22 °C. Data represent the mean value of the chemotaxis index ± standard error. Capital letters denote statistically significant differences ($p < 0.05$) among various VOCs at the same concentration within a single nematode species. Lowercase letters indicate statistically significant differences ($p < 0.05$) among the different nematode species for the same VOC and VOC concentration. The VOCs used in the experiment are as follows: DMS = dimethyl sulfide, DMDS = dimethyl disulfide, AITC = allyl isothiocyanate, PEITC = phenylethyl isothiocyanate, BN = benzonitrile, and control = 96% ethanol. The chemotaxis index values are categorized as follows: values ≥ 0.2 are considered as an attractant, values between 0.2 and 0.1 are classified as a weak attractant, values between 0.1 to −0.1 have no effect, values between −0.1 to −0.2 are categorized as a weak repellent, and values ≤ −0.2 are considered as a repellent to nematodes.

VOCs	22 °C		
	Pure Compound		
	Phasmarhabditis papillosa	***Oscheius myriophilus***	***Oscheius onirici***
AITC	0.00 ± 0.00 Ba	0.00 ± 0.00 Aa	0.00 ± 0.00 Aa
BN	0.10 ± 0.02 Cb	0.08 ± 0.03 Cb	0.00 ± 0.01 Aa
DMDS	0.13 ± 0.03 Cb	0.03 ± 0.02 BCa	0.02 ± 0.01 Aa
DMS	−0.06 ± 0.03 Aa	0.01 ± 0.01 ABb	0.07 ± 0.01 Bc
PEITC	−0.03 ± 0.01 Aa	0.04 ± 0.02 BCb	−0.01 ± 0.02 Aa
Control	0.01 ± 0.01 Ba	0.01 ± 0.01 ABa	0.01 ± 0.01 Aa

Table 4. *Cont.*

VOCs	22 °C		
	Pure Compound		
	Phasmarhabditis papillosa	***Oscheius myriophilus***	***Oscheius onirici***
	0.03 ppm		
AITC	−0.04 ± 0.02 Aa	0.01 ± 0.02 Ab	0.03 ± 0.01 Bb
BN	0.05 ± 0.03 CDb	0.02 ± 0.02 ABab	0.00 ± 0.01 Aa
DMDS	0.02 ± 0.02 Ba	0.07 ± 0.02 Bb	0.01 ± 0.01 ABa
DMS	0.06 ± 0.01 Db	0.01 ± 0.01 Aa	0.07 ± 0.01 Cb
PEITC	0.00 ± 0.01 Ba	0.02 ± 0.03 ABa	−0.01 ± 0.02 Aa
Control	0.01 ± 0.01 BCa	0.01 ± 0.01 Aa	0.01 ± 0.01 ABa

At the lower VOC concentration (0.03 ppm), DMS emerged as a weak attractant for *P. papillosa* (CI = 0.06 ± 0.01) and *O. onirici* (CI = 0.07 ± 0.01), with minimal responses observed for *O. myriophilus* (CI = 0.01 ± 0.01). DMDS maintained a weak attractant effect for *O. myriophilus* (CI = 0.07 ± 0.02), while responses for *P. papillosa* and *O. onirici* were subdued. BN was a weak attractant for *P. papillosa* (CI = 0.05 ± 0.03) but elicited negligible responses for *O. myriophilus*, and *O. onirici*. AITC and PEITC displayed minimal to no effect on chemotaxis across all species, with CI values close to zero (Table 4).

4. Discussion

The chemotactic behavior of parasitic nematodes in response to volatile organic compounds (VOCs) provides critical insights into belowground multitrophic interactions and their potential applications in sustainable pest management. This study evaluated the responses of *P. papillosa*, *O. myriophilus*, and *O. onirici* to VOCs emitted by *B. nigra* roots following herbivore attack. The results highlight the species-specific and temperature-dependent nature of nematode responses to chemical cues, reinforcing the ecological complexity of plant–nematode interactions.

Among the tested VOCs, benzonitrile (BN) consistently exhibited the strongest chemoattractant properties across both temperature conditions, eliciting significant positive responses in *O. myriophilus* and *O. onirici*. This supports previous studies demonstrating the role of specific VOCs in mediating nematode attraction to plant hosts and soil environments [11–15,28]. The ability of BN to attract beneficial nematodes suggests its potential for enhancing biological control strategies in pest management. Conversely, allyl isothiocyanate (AITC) exhibited strong nematicidal properties at higher concentrations, completely inhibiting nematode motility and causing mortality in all tested species. These findings exemplify the dual role of VOCs as either attractants or toxins, illustrating the sophisticated chemical strategies plants employ to recruit beneficial organisms while deterring herbivores or pathogens [14,15,29].

The differential responses of nematodes to VOCs are particularly relevant for the management of plant-parasitic nematodes. Glucosinolate breakdown products, including isothiocyanates and nitriles, have been shown to influence root-knot nematodes (*Meloidogyne* spp.), among the most economically damaging nematodes in agriculture [30,31]. For example, Anastasiadis and Karanastasi [31] demonstrated that incorporating fresh organic amendments such as broccoli, cabbage, and cauliflower into soil significantly reduced *Meloidogyne incognita* and *M. javanica* populations. The study highlighted that volatiles released from decomposing *Brassica* residues had strong nematicidal effects, particularly when amendments were applied at higher concentrations or covered with plastic to retain volatiles. Notably, broccoli residues had the highest nematode suppression efficiency, re-

inforcing the importance of species-specific differences in VOC composition and potency. These findings underscore the potential for VOC-based approaches to not only suppress plant-parasitic nematodes but also optimize the recruitment of beneficial nematodes in pest control strategies.

Temperature significantly influenced nematode chemotaxis. At 18 °C, BN and dimethyl disulfide (DMDS) exhibited heightened attractant properties, whereas responses at 22 °C were more variable. This temperature-dependent chemotactic behavior likely reflects physiological adaptations in nematodes, affecting their ability to locate hosts and respond to chemical gradients [3,10,14,19]. Such findings are crucial for the development of VOC-based pest management, as they emphasize the need to consider environmental conditions when applying these compounds in agricultural systems [30,32]. Understanding how temperature influences nematode behavior could enhance the precision of VOC application in field conditions, ensuring maximum effectiveness [33].

The species-specific interactions between nematodes, VOCs, and environmental factors further highlight the complexity of belowground ecological networks. *O. myriophilus* and *O. onirici* demonstrated a stronger attraction to BN and dimethyl sulfide (DMS) compared to *P. papillosa*, suggesting differences in chemosensory mechanisms and ecological niches. These results align with previous research showing that nematodes exhibit distinct chemical preferences, shaped by evolutionary adaptations and environmental cues [3,10,12,14,15]. Moreover, interactions between VOCs and temperature, as well as VOCs and concentration, reinforce the need for multi-factorial assessments when designing pest management strategies.

The findings of this study provide valuable insights into the potential integration of VOCs into sustainable agricultural practices. The strong chemoattractant properties of BN suggest its potential as a biocontrol enhancer, improving the efficacy of nematode-based pest management. Conversely, the nematicidal effects of AITC highlight its potential as a direct control agent. However, several challenges must be addressed before large-scale field implementation. One of the key considerations is the stability and persistence of VOCs in soil environments. VOCs can rapidly degrade or diffuse depending on soil composition, microbial activity, and moisture levels [32]. Studies have shown that the effectiveness of VOC-based amendments diminishes over time, as demonstrated in research on *Brassica* residue applications, where nematicidal effects decreased after two to three weeks due to VOC volatilization and microbial breakdown [31]. Future research should assess the longevity of these compounds under different soil and climatic conditions to optimize application timing and frequency.

Additionally, the effects of VOCs on non-target organisms require further investigation. While certain compounds attract beneficial nematodes, they may also inadvertently influence other soil biota, potentially disrupting natural predator-prey dynamics [32]. A more comprehensive ecological assessment of VOC-based pest management approaches is needed to ensure compatibility with existing biological control programs. Lastly, climate variability and temperature fluctuations could impact the efficacy of VOC-based strategies. Given the temperature-dependent chemotactic responses observed in this study, future research should explore the adaptability of these approaches under changing climate conditions. Understanding how temperature fluctuations influence nematode behavior, VOC emissions, and overall pest dynamics will be critical for developing resilient and adaptive management strategies.

5. Conclusions

This study enhances our understanding of the chemotactic behavior of parasitic nematodes in response to volatile organic compounds (VOCs) and environmental factors,

highlighting species-specific and temperature-dependent responses. Benzonitrile emerged as a strong chemoattractant, while allyl isothiocyanate exhibited potent nematicidal effects, demonstrating the dual role of VOCs in modulating nematode behavior. These findings underscore the potential of VOC-based strategies for sustainable pest management, reducing reliance on chemical pesticides while promoting ecological balance.

However, as a preliminary investigation, this study has certain limitations. Future research should explore VOC interactions in combinations, assess nematode responses to plant-emitted versus synthetic compounds, and evaluate the direct toxicity of these compounds. Additionally, further studies should examine VOC persistence in soil, their interactions with other environmental factors, and their applicability under changing climatic conditions. Addressing these aspects will refine the practical use of VOC-based strategies and enhance their potential for sustainable agriculture and nematode control.

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