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Inhibitory Effects of 3-Octanone and 1-Octen-3-ol on Juvenile Survival, Egg Development, and Egg-Mass Hatching in *Meloidogyne* Species

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Abstract

Root-knot nematodes (RKNs) of the genus *Meloidogyne* are major plant pests causing severe crop losses. Microbial volatile organic compounds (VOCs) have emerged as promising biopesticides. In this study, we evaluated the effects of two fungal VOCs, 1-octen-3-ol and 3-octanone, on nematode survival in five *Meloidogyne* species (*M. incognita*, *M. javanica*, *M. hapla*, *M. arenaria*, and *M. luci*) in plate assays. Results showed near-complete (95–100%) J2 mortality at 500–1000 ppm within 24 h. At lower concentrations, mobility declined, and species-dependent differences were observed: 1-octen-3-ol was more effective against *M. arenaria*. Meanwhile, 3-octanone showed stronger effects only on *M. hapla* and moderate effects on *M. incognita* and *M. javanica*. Further experiments using solely *M. javanica* showed that egg differentiation was significantly inhibited at 7, 14, and 21 days, with up to an 80% reduction at 1000 ppm, and the effects persisted at 125 ppm. Egg hatching from egg masses was reduced by up to 95% in a concentration-dependent manner, irrespective of compound type. Soil-like microcosm assays resulted in substantial reductions in recovered juveniles, with over 90% reduction at 125 ppm after 24 h, suggesting sustained effects under the tested conditions. In more complex plant–soil greenhouse conditions, effects were reduced, although decreasing trends in nematode infection were observed. Overall, these results indicate that fungal VOCs exhibit strong effects on different nematode life stages under controlled conditions, highlighting 1-octen-3-ol and 3-octanone as promising candidates for further evaluation in nematode management strategies.

Keywords: biopesticide; *Meloidogyne*; nematicide; volatile organic compounds (VOC)

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1. Introduction

Plant-parasitic nematodes (PPNs) cause substantial damage to a wide range of crops, with global yield losses estimated at $157 billion annually [1–3]. Among them, root-knot

nematodes (RKN) like *Meloidogyne* spp. rank among the top ten most destructive plant-parasitic nematodes [4], infecting over 3000 plant species, including vegetables (e.g., tomato, potato), legumes (e.g., soybean), cereals (e.g., maize), and fruits (e.g., banana, melon) [5,6]. Second-stage juveniles (J2s) penetrate plant roots, inducing gall formation and disrupting root function [7], with symptoms and severity varying by species [5,6,8–10]. Among the 98 known *Meloidogyne* species, *M. arenaria*, *M. incognita*, *M. javanica*, and *M. hapla* are the most widespread and devastating for agriculture [1,4,5,7,11]. *Meloidogyne luci* is also considered an extremely damaging pest with a wide host range and has been included on the European and Mediterranean Plant Protection Organization Alert List of harmful organisms [12–14].

Managing RKNs remains a significant agricultural challenge [4–7]. Currently, various preventive strategies, including cover crops, crop rotations, soil solarization, and resistant cultivars, have been widely implemented to reduce nematode infestations [15,16]. Despite these efforts, synthetic nematicides remain a primary tool for managing RKN in heavily affected fields [3,9,16]. However, the implementation of regulatory restrictions, such as Directive 91/414/EEC and Regulation 1107/2009/EC of the European Union, has led to the withdrawal of many chemical nematicides due to environmental and human health concerns [17–19]. In addition to regulatory restrictions, the long-term efficacy of chemical nematicides is declining due to the development of nematode resistance, further limiting control options [20,21]. Microbial biopesticides have been used as nematicides [22–24], but recent research efforts are increasingly focused on natural product-based nematicides as environmentally friendly alternatives [25–28].

Plant- and microorganism-derived volatile organic compounds (VOCs) have emerged as promising biopesticides [29]. These compounds primarily act as fumigants and have been explored in various biological control applications, exhibiting nematicidal, insecticidal, and antimicrobial properties [29,30]. Certain VOCs disrupt nematode behavior and induce plant defenses, as well as acting as fumigants, offering a viable alternative to conventional nematicides [28,31–34].

Among these, the VOCs 1-octen-3-ol and 3-octanone have shown a high effectiveness as biopesticides and biostimulants. These compounds, produced by both plants and fungi, such as the entomopathogenic fungus *Metarhizium brunneum*, have been shown to promote plant growth [35,36], activate plant defenses [37], attract and kill molluscs [38], and exhibit fumigant toxicity against major soil pests such as *Agriotes lineatus* (wireworm), *Diabrotica virgifera* (corn rootworm), and *Phyllopertha horticola* (garden chafer), with responses varying greatly in a dose-dependent manner [35,39]. Studies suggest that high concentrations of these VOCs influence the behavior and survival of entomopathogenic nematodes (EPNs), potentially reducing their efficacy in biological control [40]. Furthermore, *M. hapla* has been shown to alter its behavior in response to these compounds, exhibiting attraction at low doses and repellency at higher doses [41].

The reported biological activity of these VOCs suggests they may represent promising candidates for new bionematicides. However, comparative studies evaluating their efficacy across multiple *Meloidogyne* species and life stages remain limited. This study investigates the nematicidal activity of these compounds against five major *Meloidogyne* species, focusing on their effects on juvenile survival and behavior, as well as on egg differentiation and hatching in *M. javanica*. Their efficacy was further evaluated under soil-like and plant–soil greenhouse conditions using *M. javanica*.

2. Materials and Methods

2.1. Nematode Cultures

Five species of *Meloidogyne* spp. were used in these experiments, i.e., *M. incognita*, *M. luci*, *M. arenaria*, *M. javanica*, and *M. hapla*. All *Meloidogyne* species used in the present

study were maintained as established laboratory cultures for many years under greenhouse conditions (25–30 °C, 16 h photoperiod) in Crete, Greece, and have been routinely used in previous studies. For this work, these populations were multiplied on tomato plants (*Lycopersicon esculentum* Mill. cv. Belladona) at the five-leaf stage, grown in 18 cm diameter 1 L plastic pots containing a 50:50 mixture of peat and perlite. The plants were kept in a growth chamber at 25 ± 2 °C, with 65% RH and 16 h photoperiod at the Agricultural University of Athens, Greece. After 40 days, the plants were removed from the pots, and the roots were carefully washed to remove soil residues. Egg extraction was performed using the hypochlorite method [42], with a 1% sodium hypochlorite solution, and second-stage juveniles (J2s) were obtained by placing eggs on a Baermann funnel at ambient temperature (27 ± 1 °C). J2s collected during the first three days were discarded, while newly hatched juveniles were collected every two days and used in the experiments. These procedures follow standard nematological methods for the culture, extraction, and collection of *Meloidogyne* spp. [42,43].

2.2. Nematode Mortality Bioassays

In vitro assays were conducted to assess the effects of the two VOCs on nematode mobility and viability, on the five *Meloidogyne* species. The experiments were conducted using 24-well polystyrene plates (Nunc, Thermo Fisher Scientific, Roskilde, Denmark), similarly to other studies [28,44,45]. The VOCs, 1-octen-3-ol (CAS number 3391-86-4; Sigma-Aldrich, St. Louis, MO, USA) and 3-octanone (CAS number 106-68-3; Sigma-Aldrich, St. Louis, MO, USA), were separately mixed with ethanol (20 µL of compound to 100 µL ethanol) and brought to 1 mL with 0.3% *v/v* Tween-20 aqueous solution, to create the stock solution (20,000 ppm, v/v). Concentrations were prepared volumetrically and are expressed in ppm (v/v), where 1 ppm is equivalent to 1 µL L^{-1}. Nematodes exposed to these concentrations of ethanol and Tween-20 were not affected, as preliminary tests and previous work indicate [28,44,45]. For each nematode species and VOC concentration, five replicate wells were used, each containing approximately 50 nematodes and exposed to final compound concentrations of 1000, 500, 250, or 125 ppm. The final compound solutions and nematode suspensions were mixed in each well at a 1:1 (v/v) ratio. Negative control treatments contained less than 0.1% ethanol and 0.3% Tween-20 aqueous solution. To minimize VOC evaporation, all plates were sealed with parafilm and covered with lids. The entire assay was repeated twice to ensure reproducibility.

Nematode viability and mobility were assessed and recorded at 24 h. Observations were performed under an inverted microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) at 40× and 100× magnifications. The assessment of nematode posture, motility, and viability was performed using standard nematological criteria. Individuals were initially screened based on posture; straight (I-shaped) nematodes were recorded as potentially non-motile, while curved or bent individuals (e.g., J-, L-, or S-shaped) were considered motile. To distinguish between true mortality and temporary paralysis (nematostatic effects), all non-motile individuals were subjected to mechanical stimulation using a fine probe [45,46]. Individuals that remained completely unresponsive to repeated physical stimuli were classified as non-viable, whereas those exhibiting any movement were recorded as viable. This ensures that there was no overestimation of mortality by including paralyzed individuals in the dead count.

J2 mortality data were analyzed using a univariate general linear model (GLM), with compound, species, and concentration included as fixed factors in a full factorial design. All two-way and three-way interaction terms were included in the model. Pairwise comparisons of estimated marginal means were performed using the Bonferroni correction to adjust for multiple testing. Homogeneity of variance was assessed using Levene's test,

and heteroskedasticity was additionally examined using the SPSS test for heteroskedasticity. All analyses were performed in IBM SPSS Statistics (version 30), and statistical significance was set at $p < 0.05$.

2.3. Egg Development Assay

In this assay, only *the M. javanica* species was used. Eggs were extracted from infected tomato roots (*Solanum lycopersicum* cv. Belladona) using the sodium hypochlorite method described by Hussey and Barker [42]. The egg suspension was passed through 53 µm and 38 µm sieves, thoroughly rinsed with tap water, and collected in a 100 mL beaker. Egg concentration was estimated under an inverted microscope (100×), and the suspension was adjusted to 100 eggs/mL for use in the bioassay.

The effect of 1-octen-3-ol and 3-octanone on the egg differentiation of *M. javanica* was evaluated at four concentrations (125, 250, 500, and 1000 ppm). Both compounds were prepared following previously described procedures and mixed to achieve a 1:1 (*v*/*v*) ratio with egg suspensions in Cellstar® 24-well plates (Greiner Bio-One, Frickenhausen, Germany). Each well received 0.5 mL of egg suspension (approximately 50 eggs, of which 80–90% were undifferentiated, day 0), followed by 0.5 mL of the test solution. Negative controls consisted of either distilled water or water with ethanol and Tween-20 at concentrations equivalent to those used in treatments. All plates were sealed with lids to prevent evaporation and incubated at 26 ± 1 °C.

Egg differentiation within the egg as well as hatching were monitored under an inverted microscope (100×) at 7, 14, and 21 days post-exposure. To account for natural variation, hatching rates (%) were calculated for each replicate based on the number of eggs that differentiated relative to day 0 [47,48]. Five replicates were used for each concentration, and the whole experiment was conducted four times.

Data were analyzed using a univariate general linear model (GLM), with compound, day, and concentration included as fixed factors in a full factorial design. All two-way and three-way interaction terms were included in the model. Pairwise comparisons of estimated marginal means were performed using the Bonferroni correction to adjust for multiple testing. Homogeneity of variance was assessed using Levene's test, and heteroskedasticity was additionally evaluated using the SPSS heteroskedasticity test. All analyses were performed using IBM SPSS Statistics (version 30), and statistical significance was set at $p < 0.05$.

2.4. Egg Hatching from Egg Masses Assay

Egg hatching refers to the emergence of J2s from the eggs, which, in the case of egg masses, involves both completion of embryogenesis and successful exit from the gelatinous matrix. Mature egg masses of *M. javanica* were carefully collected from washed tomato roots and placed individually in 6 cm sterile polystyrene Petri dishes used as extraction trays (Sigma-Aldrich, St. Louis, MO, USA), with one egg mass per tray [44,49]. Each egg mass was exposed to 10 mL of either 1-octen-3-ol or 3-octanone at one of four concentrations (125, 250, 500, and 1000 ppm). Test solutions of 1-octen-3-ol and 3-octanone were prepared as described previously by dissolving each compound in ethanol and bringing them to volume with a Tween-20 aqueous solution.

Egg masses remained submerged in the treatment solutions for seven days at 26 ± 1 °C. Control treatments consisted of water with ethanol and Tween-20 at concentrations used for compound dilution. After this period, solutions were discarded, and the egg masses were gently rinsed twice with clean water to remove residual VOC before being transferred to fresh trays containing 10 mL of distilled water. The trays were covered to prevent evaporation and maintained at 26 ± 1 °C. Juveniles (J2) that hatched were counted and

removed at weekly intervals. After four weeks, the experiment was terminated (hatching was not observed in control samples). Each egg mass was transferred to a microscope slide, gently crushed under a coverslip, and the number of unhatched eggs was recorded using an inverted microscope. Each treatment was replicated four times, and the experiment was independently repeated twice, yielding 8 replicates per treatment.

Data were analyzed using a univariate general linear model (GLM) with compound and concentration as fixed factors in a full factorial design. Pairwise comparisons of estimated marginal means were performed using the Bonferroni correction to account for multiple testing. Homogeneity of variance was assessed using Levene's test. All statistical analyses were conducted using IBM SPSS Statistics (version 30), with significance set at $p < 0.05$.

2.5. Soil-like (Microcosm) Assay

This assay evaluated the effects of 1-octen-3-ol and 3-octanone under soil-like conditions. This microcosm experiment was set up using sand-filled 50 mL Falcon tubes and the *M. javanica* species. The sand was not sterilized before use to better simulate soil-like conditions. The sand was moistened with the respective treatment solution to saturation to ensure uniform distribution of the compounds, and the same volume of solution was applied across all treatments to maintain consistent moisture conditions. The experimental setup was adapted from established soil-microcosm protocols used to evaluate the efficacy of volatile nematicides [49,50]. Tubes were filled with 80 g of wet sand (equivalent to 60 g of dry sand), and an aqueous suspension containing approximately 500 *M. javanica* nematodes was injected into each tube. Compound solutions were premixed with the test compound diluted in ethanol (as previously described) and serially diluted in a 0.3% Tween-20 aqueous solution to reach final concentrations. Negative controls contained less than 0.1% ethanol and 0.3% Tween-20 aqueous solution.

After 24 h, nematodes were extracted using the Baermann funnel technique, and their numbers were recorded under a microscope after 48 h. Concentrations of 1000, 500, 250, and 125 ppm were tested, each with five replicates. The experiment was repeated twice to ensure reproducibility.

Statistical analysis was performed to evaluate the effect of the volatile compounds on J2 recovery. Due to the presence of zero variance in high-concentration treatments (100% suppression of nematode recovery), the non-parametric Kruskal–Wallis test was used to assess differences among treatments (control, 1-octen-3-ol, and 3-octanone) at each concentration level. Pairwise comparisons were performed using Bonferroni-adjusted tests. Statistical significance was set at $p < 0.05$.

2.6. Plant–Soil Assay Under Greenhouse Conditions

Following the initial in vitro and soil-like assays, the effects of the volatile compounds were further evaluated in a plant–soil system under greenhouse conditions to better approximate biologically relevant exposure scenarios. This system represents a more open and complex environment, in which volatile organic compound diffusion, adsorption, and degradation are expected to reduce effective concentrations relative to controlled laboratory conditions.

An experimental setup consisting of two concentric tubes was used (Figure 1). The outer tube was filled with sand premixed with each tested compound at a concentration of 1000 ppm, as described in Section 2.5. A second inner tube, with its base removed and fitted with a mesh, was placed inside the outer tube and filled with soil (Figure 1). The mesh allowed diffusion of the VOCs from the treated sand into the soil while preventing direct contact between the compound source and plant roots.

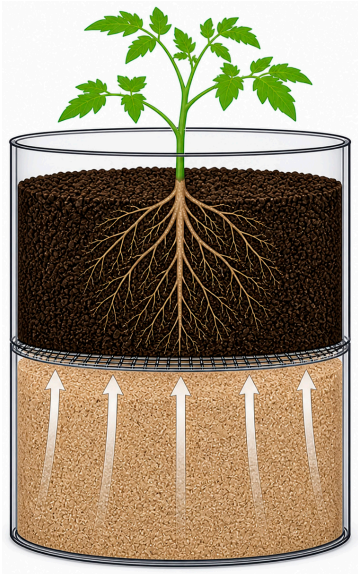


Figure 1. Schematic representation of the dual-tube system used in the plant–soil assay. Two stacked tubes of equal diameter were used, with the lower tube containing sand premixed with the volatile compound (1000 ppm) and the upper tube containing soil and a tomato seedling (*Solanum lycopersicum* cv. Belladona). Approximately 500 juveniles (J2) of *M. javanica* were introduced into the soil following transplantation. A mesh barrier between the compartments allowed diffusion of volatile compounds into the soil (arrows indicate the direction of VOCs) while preventing direct contact with plant roots.

Tomato seedlings (*Solanum lycopersicum* cv. Belladona) of approximately 14–20 days old were used. Plants were maintained under greenhouse conditions (25 ± 2 °C, ~65% RH, 16 h photoperiod) and grown in soil-filled pots before transplantation into the experimental setup. Before transplantation, seedlings were visually inspected to ensure adequate root development, with roots extending beyond the soil plug. Tomato seedlings were then transplanted into the inner tube, and approximately 500 s-stage juveniles (J2) of *M. javanica* were introduced into the surrounding soil. Plants were maintained under greenhouse conditions for 30 days. At the end of the experimental period, nematode infection and plant growth parameters were assessed. Infection levels were quantified by counting root galls containing sedentary adult females (hereafter referred to as "female counts"). Each treatment consisted of nine biological replicates.

Data from the greenhouse experiment were analyzed using a generalized linear model (GLM) with a negative binomial distribution to account for overdispersion in count data. Treatment was included as a fixed factor. Estimated marginal means were calculated, and pairwise comparisons were performed where appropriate. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Effect of VOCs on Nematode Mortality

The effects of compound, species, and concentration on J2 mortality were assessed using a univariate general linear model. Results showed that all three main factors had a significant effect on mortality (Table 1). In addition, all two-way interactions (compound × species, compound × concentration, species × concentration) and the three-way interaction (compound × species × concentration) were significant (all $p < 0.001$), indicating that the effect of each VOC depended on both nematode species and concentration (Table 1). The model explained 99.4% of the variance in mortality ($R^2 = 0.99$; adjusted $R^2 = 0.99$). However, not all pairwise differences were statistically significant (Supplementary Data).

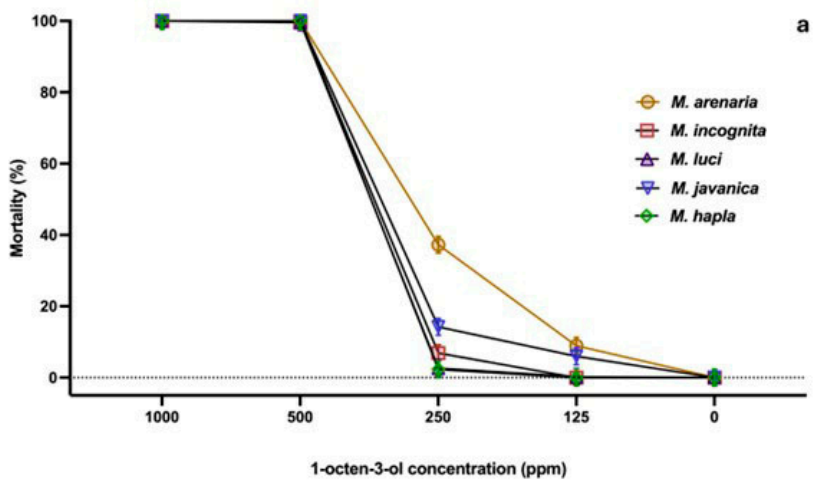
Both 1-octen-3-ol and 3-octanone significantly reduced nematode survival in plate assays at higher concentrations (500 and 1000 ppm), resulting in near-complete (96–100%) mortality within 24 h across all five *Meloidogyne* species (Figure 2a,b). Even at lower concentrations, although mortality was reduced, nematodes exposed to either compound appeared visibly less active compared to untreated controls. Differences in response among species in sensitivity and compound performance were observed (Figure 2a,b, Table 1). However, responses partially overlapped among species, indicating that not all pairwise differences were statistically distinct (Supplementary Data).

In *M. arenaria*, 1-octen-3-ol was significantly more effective than 3-octanone at 250 ppm, producing an average mortality of 37% versus 16% for 3-octanone ($p < 0.001$). At 125 ppm, 1-octen-3-ol still outperformed 3-octanone by approximately 8%, while 3-octanone did not differ significantly from the control (Figure 2). In contrast, *M. hapla* responded much more strongly to 3-octanone. At 250 ppm, 3-octanone induced 85% mortality, while 1-octen-3-ol had almost no effect (2%)—a highly significant difference ($p < 0.001$). At 125 ppm, neither compound caused notable mortality, but impaired nematode movement was evident within 24 h. Similar but less pronounced patterns were observed in *M. javanica* and *M. incognita*. For *M. javanica*, 3-octanone at 250 ppm caused 25% average mortality—significantly higher than 1-octen-3-ol (14%, $p < 0.001$). At 125 ppm, only 1-octen-3-ol showed minor lethality (~5%). In *M. incognita*, 3-octanone led to 20% mortality at 250 ppm, roughly twice that of 1-octen-3-ol (11%, $p < 0.001$). However, responses to 3-octanone were variable across replicates (e.g., 21% vs. 4.87%), while 1-octen-3-ol maintained more consistent but lower effects. Finally, in *M. luci*, both compounds caused low mortality at 250 ppm, with no significant difference between treatments or compared to control ($p = 0.811$). At 125 ppm, neither compound induced significant lethality, although treated nematodes again displayed reduced activity (Figure 2).

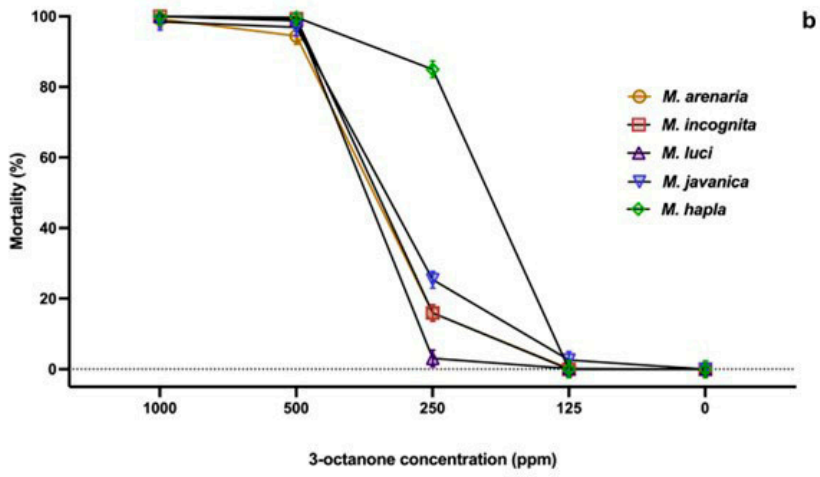
Table 1. Summary of statistical analysis results testing the effects of compound type, nematode species, and VOC concentration on nematode percent mortality. The model includes main effects (Compound, Species, Concentration), all possible two-way interactions, and the three-way interaction. All factors and interactions were statistically significant ($p < 0.001$). The model explained 99.40% of the total variance ($R^2 = 0.99$; Adjusted $R^2 = 0.99$).

Source	df	*F*	Significance (*p*-Value)
Corrected Model	49	1575.52	<0.001
Intercept	1	69,038.09	<0.001
Compound	1	46.77	<0.001
Species	4	71.27	<0.001
Concentration	4	18,277.21	<0.001
Compound × Species	4	132.86	<0.001
Compound × Concentration	4	111.32	<0.001
Species × Concentration	16	69.78	<0.001
Compound × Species × Concentration	16	104.15	<0.001
Error	450		
Total	500		
Corrected Total	499		

$R^2 = 0.99$; Adjusted $R^2 = 0.99$.



Concentration (ppm)	*M. arenaria*	*M. incognita*	*M. luci*	*M. javanica*	*M. hapla*
1000	100 (97.7–102.3)	100 (97.70–102.30)	100.0 (97.70–102.30)	100 (97.70–102.30)	100.0 (97.7–102.3)
500	100 (97.70–102.30)	99.6 (97.3–102)	99.60 (97.30–101.90)	100.0 (97.70–102.30)	100.0 (97.7–102.3)
250	37.20 (34.90–39.60)	6.9 (4.5–9.2)	2.60 (0.3–5)	14.2 (11.90–16.60)	2.3 (0.0–4.6)
125	8.90 (6.60–11.30)	0.0 (0.0–2.3)	0.20 (0–2.50)	6.0 (3.6–8.30)	0.0 (0.0–2.3)
0	0 (0–2.30)	0.0 (0.0–2.3)	0 (0–2.30)	0.0 (0.0–2.3)	0.0 (0.0–2.3)



Concentration (ppm)	*M. arenaria*	*M. incognita*	*M. luci*	*M. javanica*	*M. hapla*
1000	99.3 (96.9–101.6)	100.0 (97.7–102.3)	100.0 (97.7–102.3)	98.5 (96.2–100.8)	100.0 (98.0–102.3)
500	94.5 (92.1–96.8)	99.3 (97.0–101.6)	98.8 (96.4–101.1)	96.9 (94.6–99.3)	99.7 (97.4–102.1)
250	15.9 (13.6–18.3)	15.9 (13.6–18.3)	3.1 (0.7–5.4)	25.3 (23.0–27.7)	85.0 (82.7–87.3)
125	0.2 (0–2.5)	0 (0–2.3)	0.2 (0–2.5)	2.6 (0.3–5.0)	0 (0–2.3)
0	0 (0–2.3)	0 (0–2.3)	0 (0–2.3)	0 (0–2.3)	0 (0–2.3)

Figure 2. Mean percent mortality for five *Meloidogyne* species: *M. arenaria*, *M. incognita*, *M. luci*, *M. javanica*, and *M. hapla*, 24 h after exposure to varying concentrations of (**a**) 1-octen-3-ol and (**b**) 3-octanone. Each line represents the mean mortality for a given species across concentrations, with error bars indicating 95% confidence intervals. Each treatment included five replicates, and the experiment was repeated twice (n = 10 per treatment). Statistical analysis was performed using a univariate general linear model (GLM) followed by pairwise comparisons with Bonferroni correction ($p < 0.05$). Detailed pairwise comparisons are provided in the Supplementary Data. Corresponding numerical values (mean and 95% CI) are provided in the tables below each panel.

3.2. Effect of VOCs on Egg Development

Both compounds significantly affected egg differentiation of *M. javanica* compared to controls. The inhibitory effects of the two VOCs across different concentrations and time points are shown in Figure 3a–c.

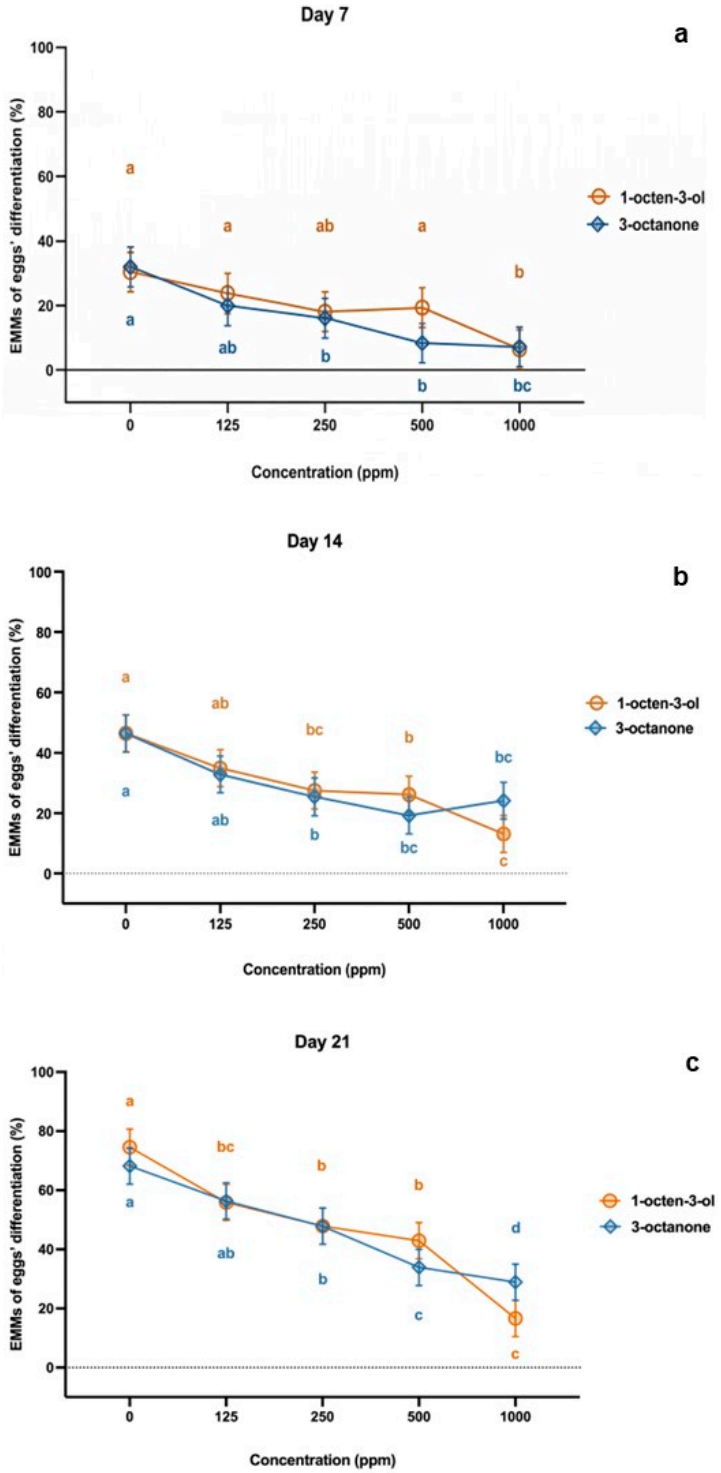


Figure 3. Mean percentage of differentiated *M. javanica* eggs after exposure to increasing concentrations of 1-octen-3-ol and 3-octanone at: (**a**) 7 days, (**b**) 14 days, and (**c**) 21 days. Differentiation rates were calculated per replicate as the percentage of eggs differentiated relative to day 0, and the graph shows the mean of these relative percentages. Error bars indicate 95% confidence intervals. Five replicates were used per treatment, and the experiment was repeated four times (n = 20 per treatment). Hatching rates generally declined with increasing compound concentration and exposure

time. Different lowercase letters indicate statistically significant differences among concentrations within each compound. Treatments that do not share a letter (within each compound) differ significantly. Data were analyzed using a univariate general linear model (GLM) followed by Bonferroni-adjusted comparisons ($p < 0.05$).

Day and concentration both had significant effects on egg differentiation, whereas the main effect of the compound was not significant (Table 2). Moreover, significant interactions between exposure time (days) and concentration, as well as between compound and concentration, suggest that each compound's effectiveness varies depending on both dose and time (Table 2). In contrast, the compound × day interaction and the three-way interaction were not significant ($p > 0.05$). The model explained 61.70% of the variance in egg differentiation ($R^2 = 0.62$; adjusted $R^2 = 0.60$). Details regarding all statistical pairwise comparisons can be found in the Supplementary Data.

Table 2. Results of the general linear model (GLM) analyzing the effects of compound, time (days), and VOC concentration on the percentage of egg differentiation in *M. javanica*. Significant main effects were observed for Day, Concentration, and the Compound × Concentration and Day × Concentration interaction terms ($p < 0.001$). However, the effect of Compound alone was insignificant ($p = 0.311$), nor were the Compound × Day or Compound × Day × Concentration interactions. This indicates that differential suppression was primarily driven by exposure duration and compound concentration, rather than compound identity alone.

Source	df	*F*	Significance (*p*-Value)
Corrected Model	29	31.63	<0.001
Intercept	1	3107.10	<0.001
Compound	1	1.03	0.311
Day	2	221.55	<0.001
Concentration	4	99.79	<0.001
Compound × Day	2	0.60	0.547
Compound × Concentration	4	5.66	<0.001
Day × Concentration	8	5.57	<0.001
Compound × Day × Concentration	8	0.72	0.671
Error	569		
Total	599		
Corrected Total	598		

$R^2 = 0.62$, Adjusted $R^2 = 0.60$.

By day 7, exposure to the highest tested concentration (1000 ppm) of either compound resulted in approximately 78% fewer differentiated eggs compared to the control. Specifically, differentiation dropped from 30.50% to 6.50% for 1-octen-3-ol and from 32.0% to 7.30% for 3-octanone. By Day 14, both compounds displayed a clear dose-dependent inhibitory effect. For 1-octen-3-ol, significant suppression began at concentrations of 250 ppm and above, culminating in a ~72% reduction at 1000 ppm. A similar pattern was observed with 3-octanone, though the overall effect was slightly less intense.

By day 21, inhibition of differentiation remained strong for both VOCs, but 1-octen-3-ol showed higher efficacy at lower doses. A significant reduction (~26%) was already evident at just 125 ppm, highlighting its greater potency over time (Supplementary Data).

3.3. Effects of VOCs on Egg Hatching from Egg Masses

Egg hatching was analyzed using a univariate general linear model including compound and concentration as fixed factors. The percentage of egg hatching from egg masses was significantly affected by the concentration, while neither the compound itself nor the interaction between compound and concentration had a significant effect (Supplementary Data). The model explained a large proportion of the variance (R^2 = 0.92; adjusted R^2 = 0.90).

Both compounds significantly reduced the percentage of hatched eggs from egg masses compared to the control, starting with reductions of 84% for 1-octen-3-ol and 75% for 3-octanone at the lowest tested dose (125 ppm), and reaching near-complete inhibition (~95% and 91%, respectively) at higher concentrations (500 and 1000 ppm) (Figure 4). 1-octen-3-ol consistently caused strong suppression across all tested concentrations (125–1000 ppm), with no statistically significant differences among doses (125 to 1000 ppm). In contrast, 3-octanone exhibited a clear dose-dependent effect: lower concentrations were statistically less effective than higher concentrations, and higher concentrations were required to achieve comparable suppression levels to the respective ones of 1-octen-3-ol (500–1000 ppm) (Figure 4) (Supplementary Data).

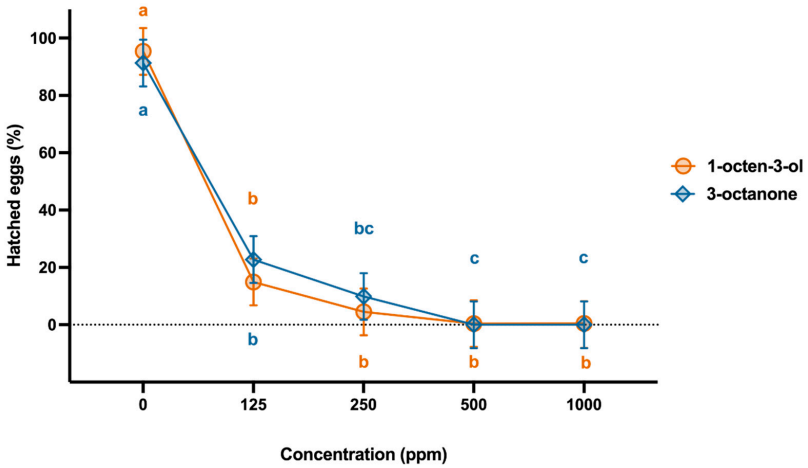


Figure 4. Effect of 1-octen-3-ol and 3-octanone concentration on egg hatching from egg masses of *M. javanica*. Estimated marginal means (EMMs) of hatched eggs of *M. javanica* are shown for each compound, with error bars indicating 95% confidence intervals. Each treatment included four replicates, and the experiment was repeated twice (n = 8 per treatment). Data were analyzed using a univariate general linear model (GLM) with compound and concentration as fixed factors, followed by Bonferroni-adjusted pairwise comparisons ($p < 0.05$). Different letters indicate statistically significant differences among concentrations within each compound.

3.4. Effect in Soil-like (Microcosm) Assay

This assay evaluated the effects of 1-octen-3-ol and 3-octanone under soil-like conditions using sand-filled falcon tubes. Treated nematodes were collected 24 h after exposure and counted after 48 h. Both compounds resulted in a complete reduction in recovered *M. javanica* J2s at the highest concentrations tested (1000 and 500 ppm) (Figure 5). At lower concentrations (125 and 250 ppm), nematode recovery was also strongly reduced compared to the control ($p < 0.05$), indicating high efficacy even at the lowest doses. Specifically, nematode recovery numbers for 3-octanone averaged 32 (93.60% reduction) and 36 (92.80% reduction), compared with ~500 in the control group (Figure 5). At both 125 and 250 ppm, 1-octen-3-ol treatments resulted in even lower recovery counts (18.60 and 23.40 on average, respectively) than 3-octanone, although these differences between compounds were not statistically significant ($p > 0.05$). (Figure 5). Reduced recovery likely reflects both mortality

and immobilization effects, as the Baermann extraction method relies on nematode motility.

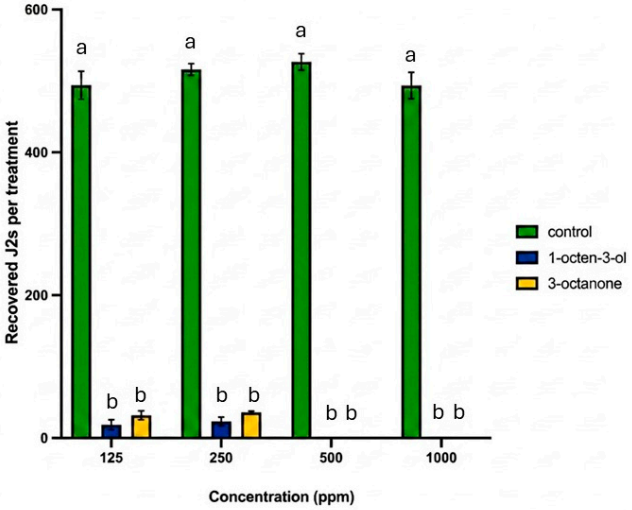


Figure 5. Number of recovered *M. javanica* J2s after exposure to 1-octen-3-ol and 3-octanone under soil-like conditions using sand-filled Falcon tubes. Nematodes were recovered 24 h after exposure using the Baermann funnel technique and counted after 48 h. Bars represent mean values, with error bars indicating 95% confidence intervals. Each treatment included five replicates, and the experiment was repeated twice (n = 10 per treatment). Data were analyzed using the Kruskal–Wallis test followed by Bonferroni-adjusted pairwise comparisons ($p < 0.05$). Different letters indicate statistically significant differences among treatments within each concentration.

3.5. Effect Under Greenhouse Plant–Soil Conditions

Under greenhouse conditions, exposure to volatile organic compounds resulted in a consistent reduction trend in nematode infection compared to the untreated control (Figure 6). Both treatments were associated with lower numbers of sedentary adult females, with incidence rate ratios (IRR) below 1 (0.74 for 1-octen-3-ol and 0.63 for 3-octanone), corresponding to estimated reductions of approximately 26% and 37%, respectively.

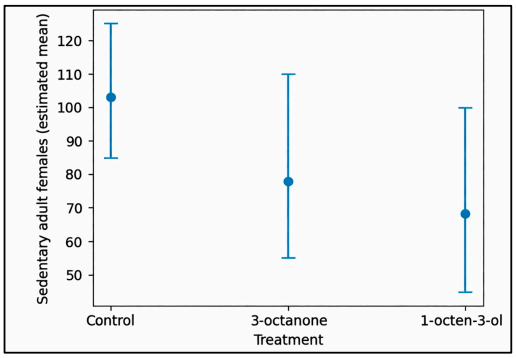


Figure 6. Estimated number of sedentary adult females per treatment under greenhouse conditions. Points represent model-estimated means derived from a negative binomial generalized linear model, and error bars indicate 95% confidence intervals. Each treatment included nine biological replicates (n = 9). Statistical analysis was performed using a negative binomial GLM, with treatment as a fixed factor. Differences between treatments were not statistically significant ($p > 0.05$). However, variability among replicates was relatively high, as reflected by the wide confidence intervals overlapping with the control, and differences were not statistically significant (negative binomial GLM, $p > 0.05$). Inclusion of root fresh weight as a covariate did not significantly affect female counts, indicating that variation in nematode infection was not associated with differences in root system size (Figure 6). Plant growth parameters were not affected by VOC exposure, as neither shoot nor root fresh weight differed among treatments, suggesting the absence of phytotoxic effects under the tested conditions.

4. Discussion

This study evaluated the effects of two volatile compounds, 1-octen-3-ol and 3-octanone, on different life stages of *Meloidogyne* species, with multi-species comparisons in juvenile mortality assays and more detailed analyses in *M. javanica* using multiple experimental approaches, demonstrating their potential as biological nematicidal agents under controlled conditions. While these VOCs have already been successfully applied against various invertebrates, including mosquitoes [51,52] and wireworms [36], as well as pathogenic microbes [53], their nematicidal properties have only recently been described [54,55].

Our findings expand this body of work by demonstrating their in vitro efficacy on J2s across five species and their efficacy on *M. javanica* populations across multiple developmental stages. The highest concentrations of 500 and 1000 ppm of both VOCs caused over 95% of J2 mortality across all species within 24 h. Lower concentrations of 250 ppm and 125 ppm were also effective, primarily impairing nematode mobility, though mortality rates differed among species. These results indicate strong activity under the tested conditions and that compound-specific effects are distinctly species-dependent. These findings align with previous research demonstrating species-dependent nematicidal effects, highlighting the importance of understanding interspecies variability when developing new formulations and their application [41,55–57]. Jang et al. [58] found that alkaloids from *Waltheria indica* exhibited differential effects on *M. arenaria* and *M. incognita*, leading to species-specific survival rates. Similarly, fluensulfone showed distinct effects on *M. arenaria* juveniles compared to *M. incognita*, eliciting different behavioral responses [59]. The observed variation further supports the notion that nematode susceptibility is influenced by genetic, morphological, and physiological factors, reinforcing the need for species-adapted biocontrol solutions [5,60–62]. These differences may also reflect variation in cuticle permeability and compound uptake, as well as species-specific detoxification capacity, which can influence sensitivity to VOC exposure. Notably, *M. hapla* differs from other species in its ability to undergo both meiotic and mitotic parthenogenesis, which may contribute to its ecological adaptability. However, the factors underlying its distinct response to the tested compounds remain unclear and require further investigation.

Egg hatching from egg masses was significantly inhibited by both VOCs in *M. javanica*, since percentages dropped sharply (over 90%) even at the lowest tested concentration (125 ppm). Several fungal and bacterial VOCs have been shown to negatively affect egg hatching on nematodes [63–65]. Ovicidal activity is considered a desirable trait for a successful commercial nematicide, since eggs are the main survival mode of *Meloidogyne* species in the soil [66,67]. Our study showed that the decrease in egg hatching, as well as egg differentiation, was strongly dependent on both concentration and exposure duration. While 1-octen-3-ol caused a higher reduction (84%) of egg hatching at 250 ppm compared to 3-octanone (75%), the differences were not significant and became marginal at the highest concentration (95 vs. 91%, respectively). This contrasts with previous studies that showed a substantially superior effect of 1-octen-3-ol on egg hatching in *M. incognita*, at the lowest concentration tested (2.5 µL) [55]. These findings may indicate that higher exposure duration or higher doses lead to converging outcomes due to accumulated toxicity, potentially masking compound-specific differences, an effect also observed in J2 mortality assays. This observation suggests that careful selection of test conditions is essential when evaluating compound-specific or species-specific responses.

The different exposure durations used across life stages reflect fundamental biological differences in nematode development and responsiveness. Second-stage juveniles (J2s) are metabolically active and respond rapidly to environmental stressors, allowing mortality and mobility effects to be assessed within short exposure periods (e.g., 24 h). In contrast,

egg development and hatching are inherently slower processes, requiring extended observation periods to capture cumulative effects on embryogenesis and emergence. The longer exposure duration of eggs and egg masses in the present study was therefore designed to assess the maximum biological impact of the tested compounds under controlled conditions. However, it is possible that such exposure durations likely exceed realistic field persistence, where VOC diffusion, adsorption, and degradation would reduce effective concentrations over time. However, previous studies have reported persistence of biological effects under specific formulation and application conditions, suggesting that the delivery strategy may play a key role in extending efficacy [35,36].

This pattern is consistent with the differences observed among (a) in vitro, (b) soil-like, and (c) plant–soil conditions, indicating that the exposure environment strongly influences apparent efficacy. In soil-like assays using sand as a carrier matrix, both VOCs resulted in over 90% reduction in recovered J2s at 125 and 250 ppm within 24 h. In this system, the compounds are distributed within the pore spaces of the sand, which likely promotes retention and localized accumulation around nematodes. This may result in more stable and sustained effective concentrations compared to liquid-based assays.

In contrast, in vitro liquid systems may facilitate more rapid diffusion and volatilization of VOCs, leading to reduced local concentrations and shorter effective exposure durations, particularly at lower doses.

However, as the Baermann funnel technique used for nematode extraction relies on motility, reduced recovery in soil-like assays may reflect both mortality and immobilization. Therefore, these reductions should be interpreted as functional suppression rather than confirmed lethality. Immobilized nematodes would be unable to migrate toward host roots, thereby limiting infection, even in the absence of immediate mortality. Alternative extraction methods, such as sucrose centrifugation, could help distinguish between mortality and immobilization and should be considered in future studies.

In contrast, under greenhouse plant–soil conditions, VOCs were not in direct contact with the nematodes present in the soil but diffused from a lower layer into the rhizosphere. Under these conditions, effects were reduced compared to the soil-like system, although consistent decreasing trends in female counts were observed. This reduced efficacy likely reflects diffusion limitations and the increased complexity of the system, where VOC adsorption to soil particles, microbial degradation, and plant-mediated interactions can substantially limit effective exposure. In addition, VOCs may be rapidly diluted within the soil matrix and subject to microbial transformation, further reducing their bioavailability. The indirect exposure configuration used in this study likely resulted in lower concentrations reaching the rhizosphere compared to direct-contact assays. In addition, the heterogeneous distribution of VOCs within the soil–plant system may contribute to variability among replicates and inconsistent local concentrations. Nevertheless, the absence of phytotoxic effects and the observed reduction trends indicate that these compounds retain biological activity even under indirect exposure conditions, although their performance appears to be strongly dependent on delivery methods and environmental conditions.

Together, these findings highlight the importance of application strategy and diffusion dynamics when evaluating VOC-based nematicides in realistic environments. They also indicate that VOC-based treatments can contribute to nematode suppression under appropriate conditions, while emphasizing the need for further soil-based investigations.

Moreover, the relative effectiveness of 1-octen-3-ol against *M. javanica* in soil-based assays contrasts with our initial findings in the in vitro plate assays, where 3-octanone caused higher levels of mortality at intermediate concentrations (e.g., 250 ppm). Similarly, while our results for *M. hapla* are consistent with the strong nematicidal effects reported by Khoja et al. (2021) [41], who observed >97% mortality within 6 h at the highest dose

tested (20 µL), Veronico et al. (2023) [55] reported lower LD_{50} values for 1-octen-3-ol (3.2 µL) compared to 3-octanone (4.6 µL), indicating higher potency of 1-octen-3-ol under fumigation conditions against *M. incognita*. These differences in compound performance across studies likely reflect differences in experimental design and exposure conditions, as previous studies were conducted in sealed fumigation systems using volumetric dosing (µL), whereas the present study employed concentration-based exposure (ppm) in liquid and soil-associated systems. Such differences influence VOC diffusion, availability, and effective exposure, and therefore limit direct quantitative comparisons.

Together, these findings suggest that the apparent efficacy of these VOCs is strongly dependent on the exposure system, with compound-specific effects varying according to environmental context and delivery method, a trend also observed in other studies [41,68].

However, the results of this study indicate that these compounds show strong nematicidal activity in plate assays and substantial suppressive effects in soil-like systems, compared with previously reported microbial VOCs under controlled conditions [29]. Bacterial VOCs (e.g., from *Pseudomonas* and *Bacillus* spp.) have been studied for their nematicidal properties against *M. incognita* and *M. javanica* [69–72]. Despite being less studied, recent research has identified fungal VOCs with nematicidal activity. For instance, Mei et al. [65] found that cyclohexanol, cyclohexanone, and cyclohexanamine, produced by *Duddingtonia flagrans*, were effective against *M. incognita* at specific concentrations. Similarly, *Fusarium oxysporum* was reported to emit 2-methylbutyl acetate, 3-methylbutyl acetate, ethyl acetate, and 2-methylpropyl acetate, which were toxic to *M. incognita*, causing 88 to 96% J2 mortality [66], as well as 70% and 65% reduction in infectivity and reproduction [63,72]. Moreover, VOCs from *Daldinia* spp. reduced viability of *M. javanica* J2s by 67%, while the synthetic mixture of VOCs showed a 99% reduction in J2 viability and 87% inhibition of egg hatching [61]. Our observed mortality rates exceeding 95% across five *Meloidogyne* species at concentrations of 500–1000 ppm, along with over 90% reductions in J2 survival under soil-like conditions, and over 90% inhibition of egg differentiation and egg hatching from egg masses at lower concentrations (125–250 ppm), highlight 1-octen-3-ol and 3-octanone as promising candidates for further development as nematicidal agents targeting multiple life stages of the *Meloidogyne* life cycle. Their effectiveness is further complemented by their widespread occurrence in nature, low cost, and transient environmental persistence. Notably, both compounds are already approved for use in the food industry as flavoring agents (e.g., FEMA GRAS status, 21 CFR 172.515), which may support their future evaluation and potential integration into crop protection strategies.

The mechanism of action of these VOCs has been recently explored by Veronico et al. [55], who showed that exposure of *M. incognita* J2 to 1-octen-3-ol and 3-octanone as fumigants in a closed environment resulted in elevated levels of ROS, causing oxidative stress, inducing damage to internal structures, and leading to death. This mechanism has been proposed to be similar to the mode of action of other nematicides [55]. If this mechanism is consistent across species, the species-specific responses observed in this study could be attributed to differences in oxidative stress tolerance or detoxification mechanisms. Supporting the idea of conserved toxicity, studies in *Drosophila* have shown that 1-octen-3-ol can also cause neurological damage by disrupting dopamine homeostasis and interacting with genetic variants in dopamine biosynthesis to enhance dopaminergic neurodegeneration [73]. In addition to their nematicidal properties, 1-octen-3-ol and 3-octanone are already known to promote plant growth in various angiosperms, many of which are also susceptible to nematode infestations [35]. By simultaneously reducing nematode populations and enhancing plant vigor, these compounds could contribute to an integrated approach to crop protection, improving overall plant health and resilience in agricultural systems.

Overall, this study provides evidence that 1-octen-3-ol and 3-octanone are VOCs with strong nematicidal activity in in vitro assays, under controlled conditions, with species-dependent effects on J2s of major *Meloidogyne* species. Their activity across in vitro, soil-like, plant–soil systems as well as across different life stages, highlights their potential as candidates for sustainable alternatives to conventional nematicides. The observed variation in species susceptibility underscores the need to further investigate the physiological and genetic factors underlying nematode responses to VOCs. In addition, careful selection of test concentrations and exposure durations is essential when evaluating nematicidal activity, as high doses or prolonged exposure may obscure compound- or species-specific differences due to cumulative toxicity. Additionally, the dual functionality of these compounds in promoting plant growth while reducing nematode recovery and mobility further supports their potential applicability in integrated pest management strategies.

Future work should focus on optimizing formulation and delivery strategies to improve the stability and persistence of these volatile compounds under field conditions. In particular, approaches such as encapsulation or controlled-release systems may enhance efficacy in soil environments. In addition, large-scale field trials are needed to evaluate their performance under realistic agricultural conditions. Finally, further studies should also aim to distinguish between nematicidal and nematostatic effects and to assess long-term impacts on nematode populations and crop performance.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae12050591/s1. Supplementary Data: Statistical analyses for all assays performed in this study.

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