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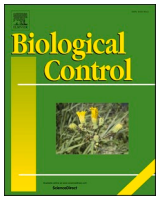

Compatibility of entomopathogenic nematodes and essential oils: A new step for integrated pest management of the truffle beetle

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HIGHLIGHTS

- *Allium sativum* EO increased EPN mortality and reduced their infective capacity.
- *Satureja montana* EO caused low mortality rates but exhibited repellency towards EPNs.
- *Mentha suaveolens* EO minimally affected EPNs survival, infectivity and reproduction.
- *M. suaveolens* EO is suggested as the most compatible oil for field use with EPNs.
- Field validation is essential to confirm the practical applicability of these findings.

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ABSTRACT

The European truffle beetle, *Leiodes cinnamomeus*, is the most important pest in black truffle (*Tuber melanosporum*) plantations. Entomopathogenic nematodes (EPNs) are promising biological control agents against *L. cinnamomeus*. Essential oils (EOs) are also recently being investigated for the control of the adults of this pest. Therefore, both control methods could be combined in Integrated Pest Management (IPM) programs to enhance their efficacy. However, limited information exists regarding the effects of the EOs on EPNs and so their compatibility. The aims of our work were to study the effects of three previously described insecticidal and nematicidal essential oils, *Allium sativum*, *Mentha suaveolens*, and *Satureja montana,* on the survival, infectivity, reproduction, and attraction behaviour of three EPN species: *Steinernema feltiae*, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*. Therefore, we conducted three experiments under laboratory conditions to observe the lethal and sublethal effects by direct contact, the lethal effect by fumigation, and the chemotaxis response. *Allium sativum* caused the highest mortality rates in all three EPN species at 24 and 72 h post application whether by direct contact (97–99%) or fumigation (40–42%), and it also reduced their infective capacity on *Galleria mellonella*. *Satureja montana* EO caused low mortality rates compared to *A. sativum*, but it was significantly more lethal (6–8%) than the control (0–3%) at 72 h in the direct contact assay. It also displayed repellent properties against *S. feltiae* and *H. bacteriohora* in the chemotaxis assay. In contrast, *M. suaveolens* EO exhibited minimal impact on the survival, infectivity and reproduction of all three EPN species. Therefore, our results suggest *M. suaveolens* oil may be the most compatible EO for use integrated with EPNs. Further validation under field conditions and in the presence of *L. cinnamomeus* is necessary to confirm the practical applicability of these findings.

1. Introduction

The European truffle beetle, *Leiodes cinnamomeus* (Panzer, 1793) (Coleoptera: Leiodidae), is the most important pest in black truffle plantations *Tuber melanosporum* Vittad., 1831 (Pezizales: Tuberaceae) in Southern Europe (Arzone, 1971); (Martín-Santafe et al., 2014) . Both

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adults and larvae feed on *T. melanosporum* fruiting bodies, causing galleries that reduce their quality and can lead to economic losses of up to 70 % in plantations (Barriuso et al., 2012). Current control methods, which rely on frequent truffle collections (Martín-Santafe et al., 2014) and the use of traps for mass capture of adults (Navarro-Llopis et al., 2021), are not efficient enough in reducing the population of *L. cinnamomeus,* so new sustainable strategies have been explored. Entomopathogenic nematodes (EPNs) belonging to the Steinernematidae and Heterorhabditidae families are obligate parasites of a wide range of insect species and hold great potential as biological control agents for different pests (Shapiro-Ilan et al., 2017). Recently, *Steinernema feltiae* (Filipjev, 1934) (Panagrolaimida: Steinernematidea), *Steinernema carpocapsae* (Weiser, 1955) (Panagrolaimida: Steinernematidea) and *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) have showed great potential as biological control agents against *L. cinnamomeus* larvae and adults under laboratory conditions (Julià et al., 2023). *Steinernema carpocapsae* induced 100 % mortality of adults at 20 °C, while *S. feltiae* caused 73 % mortality of mycophagous larvae at the same temperature. Additionally, *H. bacteriophora* resulted in a 100 % mortality of diapause larvae at 25 °C. These promising results suggest EPNs could be incorporated in Integrated Pest Management (IPM) programs to enhance the control of this pest.

These IPM programs can integrate EPNs with other biological and biorational control agents. Compatibility studies have been conducted between EPNs and other biological pest control agents, such as predators and parasites (Garriga et al., 2019). However, research on their compatibility with biorational products is limited. Bioactive compounds derived from plant extracts are promising sources of new bioactive ingredients for the development of biopesticides. These can be essential oils, non-volatile extracts, essential oil hydrolates or pure natural molecules (Gonzalez-Coloma et al., 2010).

Essential oils (EOs) are complex, biodegradable, volatile, and lipophilic mixtures of terpenoids (monoterpenoids and sesquiterpenoids) and phenylpropanoids, which are part of the plant's secondary metabolites used for defensive strategies (Mossa, 2016). Some studies have reported that the EOs' components induce neurotoxic effects in insects and nematodes through contact, ingestion, or fumigation, affecting octopamine synapses, GABA, and by inhibiting acetylcholinesterase (Pavela and Benelli, 2016); (Kesraoui et al., 2022) ; (Catani et al., 2023) . In addition, EOs may exhibit other behavioral effects, such as repellent effects, feeding and oviposition deterrence, and interference with insect growth (Mossa, 2016). These different bioactivities of EOs are directly related to their chemical composition, which can vary among populations of plant species (Angioni et al., 2006); (Barra, 2009) . Therefore, the insecticidal and nematicidal potential of various EOs has been widely demonstrated (Digilio et al., 2008); (Andrés et al., 2012) . Recently, EOs extracted from mint (*Mentha suaveolens* Ehrh., 1792 Lamiales: Lamiaceae), savory (*Satureja montana* L., 1753 Lamiales: Lamiaceae), and garlic (*Allium sativum* L., 1753 Asparagales: Amaryllidaceae) have shown nematicidal and insecticidal-acaricidal properties (Navarro-Rocha et al., 2020); (Valcárcel et al., 2021) . These EOs are also recently being investigated for the control of the adults of *L. cinnamomeus* (Sánchez-Durán, 2023).

Most research on the effects of EOs on nematodes has focused on controlling plant-parasitic nematodes (Andrés et al., 2012). However, limited information exists regarding the effects of these EOs on EPNs (Barua et al., 2020). Understanding whether EPNs and EOs can be integrated into an IPM program may enhance the control of the beetle *L. cinnamomeus*. Therefore, the aims of our research were to study the effects of three EOs, extracted from *A. sativum*, *M. suaveolens*, and *S. montana*, on *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* survival, infectivity, reproduction and attraction behavior under laboratory conditions.

2. Material and methods

2.1. Source of nematodes and essential oils

Three EPN species were used in the experiments: *Steinernema feltiae* (strain TE15), *Steinernema carpocapsae* (strain B14) and *Heterorhabditis bacteriophora* (strain CT47). These nematodes were collected from various locations of Spain: *S. feltiae* from a truffle plantation in Mora de Rubielos (Teruel), *S. carpocapsae* from an urban garden in Barcelona, and *H. bacteriophora* from a truffle plantation in Boixols (Lleida).

Nematodes were reared at 25 °C in last instar larvae of the greater wax moth, *Galleria mellonella* (L., 1758) (Lepidoptera: Pyralidae) (Woodring and Kaya, 1988). The emerged infective juveniles (IJs) were recovered using modified White traps (White, 1927) and stored at 9 °C for a maximum of two weeks. Prior to application, IJs were acclimatized at room temperature for 2 h and their viability was checked under a stereomicroscope.

Three EOs were used in the experiments, each extracted from different aromatic plant species with previously characterized chemical composition and potential bioactivity: mint (*Mentha suaveolens*) (Andrés et al., 2012), savory (*Satureja montana*) (Navarro-Rocha et al., 2020), and garlic (*Allium sativum*) (Galisteo et al., 2022).

2.2. Lethal and sublethal effects by direct contact

The lethal effect assay was conducted in 96-well plates. In each well, 300 IJs in 95 μL of water were mixed with 5 μL of EOs diluted in dimethyl sulfoxide (DMSO) with 0.6 % Tween 20 (20 μg/μL) to give a final concentration of 1 mg/mL (Andrés et al., 2012). Controls consisted of 100 μL of water and 100 μL of DMSO + Tween. The plates were sealed with parafilm to prevent moisture loss and held in a chamber at 23 °C. The mortality of IJs was assessed at 24 and 72 h post-application. At each time, a 25 μL aliquot was extracted from each well containing approximately 50 nematodes, and live and dead nematodes were counted. There were ten replicates for each treatment, and the experiments were conducted three times.

To assess the sublethal effects of EOs on the infectivity and reproduction of EPNs, a contact exposure assay was conducted at the same concentration as the previous assay. EPN suspension (1000 IJs) were placed in watch glasses along with either 1 ml of EO solution or water (control). The watch glasses were maintained in a chamber at 23 °C for one to three days, depending on the treatment applied: those containing *A. sativum* and its controls were exposed for 24 h, while those containing *M. suaveolens* and *S. montana* and their controls were exposed for 72 h. This difference in exposure time was due to the higher mortality of EPNs observed after 72 h of exposure to the first two products in the lethal effect assay compared to the two EOs. Following the exposure to EOs, the surviving IJs were used to infect larvae of *G. mellonella* to evaluate their infectivity and reproduction using 24-well plates. Each well was filled with 3 g of moistened sand (10 %, w/w), and 15 IJs were counted and placed individually into each well. Prior to application, IJs were washed three times to remove any product residues on their cuticles. Finally, one larva of *G. mellonella* was added to each well to assess EPNs infectivity. Larval mortality was checked after 48, 72, 96 and 120 h. Dead larvae were placed in Petri dishes to observe if nematodes were able to reproduce after 7–10 days. Ten replicates were performed for each treatment and EPN species, and the experiments were conducted three times.

2.3. Lethal effect by fumigation

The experiment was conducted in 96-well plates and each plate contained three types of wells: (i) Water wells with 100 μL of water to maintain humidity; (ii) EPNs suspension wells with 95 μL of nematode suspension (≈ 300 nematodes/100 μL) + 5 μL of DMSO and Tween; and (iii) treatment wells with 95 μL of water + 5 μL of essential oil (Fig. 1).

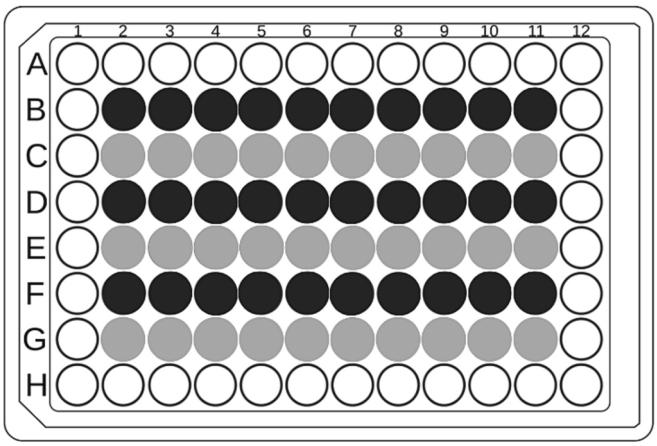

Fig. 1. Distribution of the wells content in the fumigation assay. Black wells: EPNs, grey wells: EO treatment, white wells: water.

Essential oils were used at the same final concentration as the previous assays (1 mg/mL). The water wells were distributed around the perimeter of the plate, while the treatment and EPNs wells were distributed in the six alternating rows of the plate (Fig. 1). In the case of control plates, treatment wells contained only 100 μL of water. The plates were sealed with parafilm to prevent moisture loss and stored in a chamber at 23 °C. At 24 and 72 h post application, 25 μL of the EPNs suspension were extracted from each well (≈ 50 IJs) and live and dead nematodes were counted to determine IJs mortality. There were ten replicates for each treatment and the experiments were conducted three times.

2.4. Chemotaxis assay

The chemotaxis assay was conducted based on the methodology developed by Ward (Ward, 1973) and O'Halloran and Burnell (O'Halloran and Burnell, 2003) and modified by Laznik and Trdan (Laznik and Trdan, 2016). Petri dishes with a diameter of 9 cm and filled with 25 ml of 1.2 % technical agar (Sigma) were employed. Essential oils were dissolved in ethanol obtaining a final concentration of 1 mg/mL. For each assay, a volume of 10 μL of the test substance was applied to the right side of the agar surface (treated area), while 10 μL of ethanol (solvent) was placed on the left side of the agar surface (control area). In the control treatment, 10 μL of ethanol were applied to the treated area and 10 μL of distilled water to the control area. A filter paper disc with a diameter of 1 cm was placed in the center of the agar surface (inner circle). Subsequently, 50 μL of water containing 100 IJs of either *S. feltiae*, *S. carpocapsae* or *H. bacteriophora* were applied on the filter paper. Petri dishes were placed in a dark rearing chamber at 23 °C and the number of IJs in the treatment and control areas were counted after 4 h using a binocular microscope. There were five replicates for each treatment and the experiments were conducted three times.

The specific chemotaxis index (CI) (Bargmann and Horvitz, 1991), which ranged from 1.0 (perfect attraction) to − 1.0 (perfect repulsion), was calculated using the following formula:

$$CI = \frac{\text{Number of EPN in the treatment area} - \text{Number of EPN in the control area}}{\text{Total number of nematodes in the assay}}$$

2.5. Statistical analysis

A linear model (LM) or a linear mixed model (LMM) (when the exposure time factor was considered) was used, with an arcsine square root transformation of the data, to test for significant differences in the mortality of EPNs in the lethal effect assays. A linear model (LM) was also applied to compare the attraction response of EPNs to the EOs. For the sublethal effect assay, a generalized linear model (GLM), with binomial distribution and a logit link function, was used to test for significant differences in the infectivity and reproduction of EPNs. Survival curves were also obtained using a Kaplan–Meier estimator and were compared with a log-rank test. For each assay, Main effects in the factorial design were analyzed for interactions, followed by a complete analysis of simple effects. Subsequently, Tukey's multiple range test was performed to compare differences among treatments. All data were analyzed using R software (version 4.3.1) (R Core Team, 2023). Any comparison was considered significant if the p-value was less than 0.05.

3. Results

3.1. Lethal and sublethal effects by direct contact

The lethal effect assay demonstrated only significant interactions between treatment and exposure time ($F = 137.12$, $df = 4$, $p < 0.001$). When the main effects were analyzed independently, EPN species did not significantly impact on nematode mortality ($F = 0.96$, $df = 2$, $p = 0.38$).

Only *A. sativum* EO showed significant differences on the mortality of *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* compared to controls and the other treatments after 24 h ($F = 20.65$, $df = 4$, $p < 0.001F = 23.74$, $df = 4$, $p < 0.001$ and $F = 17.91$, $df = 4$, $p < 0.001$, respectively) (Fig. 2). After 72 h, both *A. sativum* and *S. montana* EOs resulted in significantly higher mortality compared to controls ($F = 905.45$, $df = 4$, $p < 0.001$for *S. feltiae*; $F = 726.55$, $df = 4$, $p < 0.001$for *S. carpocapsae* and $F = 949.21$, $df = 4$, $p < 0.001$for *H. bacteriophora*) (Fig. 2). In fact, *A. sativum* EO was the treatment with the highest nematicide effect on the three EPN species (97.72–98.99 % mortality) (Fig. 2).

Paralysis of live nematodes was caused by *A. sativum*, *M. suaveolens* and *S.montana* EOs at 24 and 72 h post-application. At 24 h, nematodes exhibited limited movement, requiring light contact to regain mobility. This paralysis became more pronounced at 72 h, necessitating increased contact for movement.

For the sublethal effect of EOs on EPN infectivity, all three EPN species exposed to *M. suaveolens* and *S. montana* EOs did not exhibit significant differences in *G. mellonella* larvae mortality compared to the control at 48, 72, 96 and 120 h post application (Fig. 3a, 3b and 3c). In fact, 100 % mortality of *G. mellonella* larvae was observed for all three EPN species after 120 h in these two EO treatments and the control. In contrast, EPNs exposed to *A. sativum* EO showed significant differences in *G. mellonella* larvae mortality compared to the control and the other treatments after 48, 72, 96 and 120 h. *Steinernema feltiae*, *S. carpocapsae*, and *H. bacteriophora* induced mortality rates of 57 %, 40 %, and 27 %, respectively, after 120 h when exposed to *A. sativum* EO (Fig. 3a, 3b and 3c). The log rank test further confirmed that EPNs exposed to *M. suaveolens* and *S. montana* EOs did not affect the survival curves of *G. mellonella*, but only *A. sativum* treatment exhibited a significant difference from the control ($\chi^2 = 66.70$, $df = 3$, $p < 0.001$ for *S. feltiae*; $\chi^2 = 86.80$, $df = 3$, $p < 0.001$ for *S. carpocapsae*; $\chi^2 = 52.30$, $df = 3$, $p < 0.001$ for *H. bacteriophora*).

The sublethal effect on EPN reproduction revealed no significant interactions between treatment and EPN species factors ($\chi2 = 5.46$, $df = 6$, $p = 0.48$). In fact, none of the EPN species showed significant differences in their reproduction between EO treatments and the control ($\chi2 = 5.82$, $df = 3$, $p = 0.12$ for *S. feltiae*; $\chi2 = 6.13$, $df = 3$, $p = 0.11$ for *S. carpocapsae* and $\chi2 = 6.18$, $df = 3$, $p = 0.10$ for *H. bacteriophora*) (Fig. 4a, 4b and 4c, respectively).

3.2. Lethal effect by fumigation

The assay revealed a significant interaction between treatment and exposure time, with no other significant interactions detected ($F = 480.84$, $df = 3$, $p < 0.001$). When the main effects were analyzed independently, EPN species did not have a significant impact on nematode mortality ($F = 2.20$, $df = 2$, $p = 0.11$).

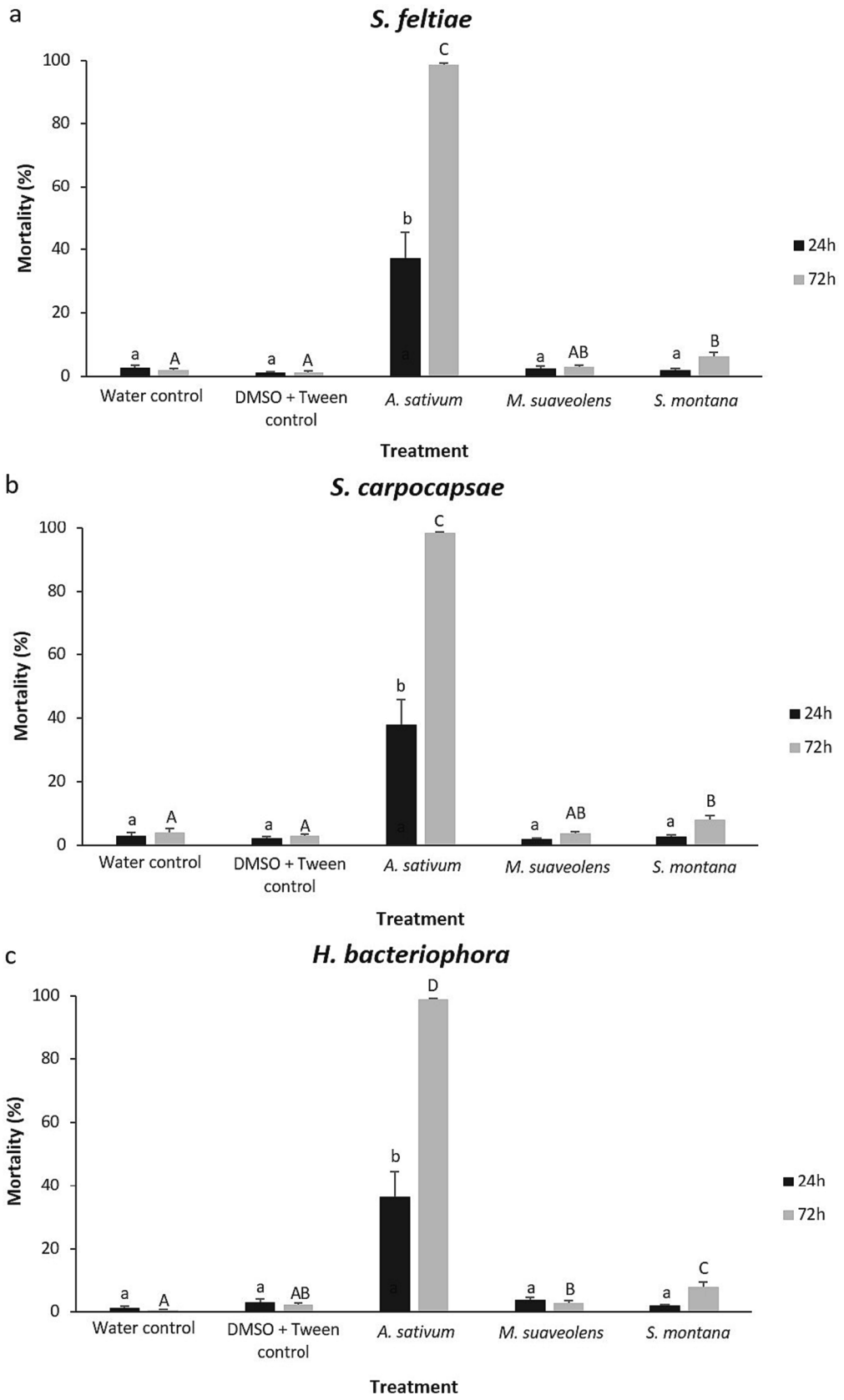

Fig. 2. Percentage of mortality (±SE) of EPNs directly exposed to three different EOs and two controls (water and DMSO + Tween) at 24 and 72 h post application. (a) *S. feltiae*, (b) *S. carpocapsae*, (c) *H. bacteriophora*. Within each column, different letters indicate significant differences among treatments for each time ($p < 0.05$).

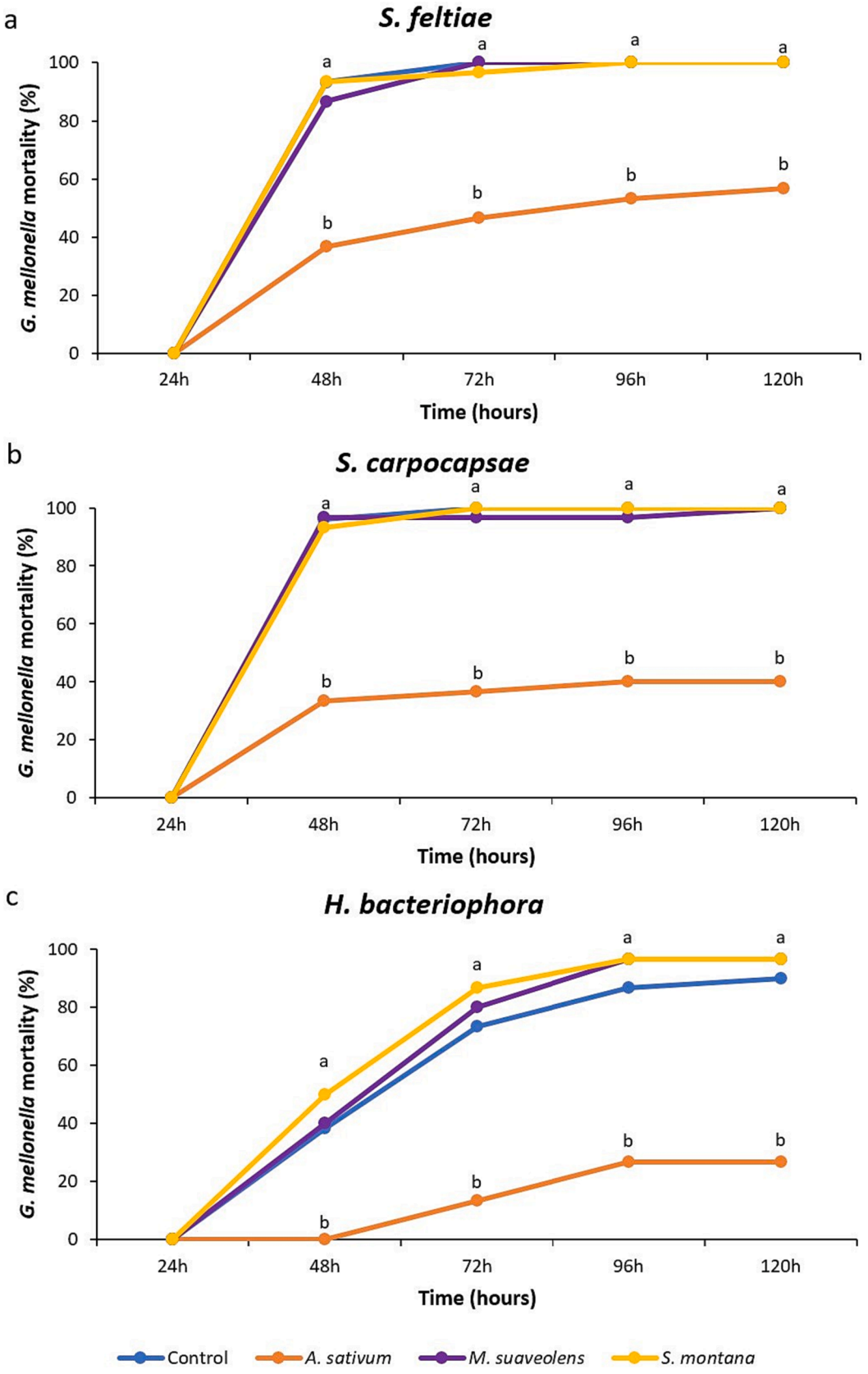

Fig. 3. Percentage of mortality (%) of *G. mellonella* larvae caused by EPNs previously exposed to EO treatments and the control at 24, 48, 72, 96 and 120 h. (a*) S. feltiae* (b) *S. carpocapsae* (c) *H. bacteriophora*. Within each time, different letters indicate significant differences among treatments ($p < 0.05$).

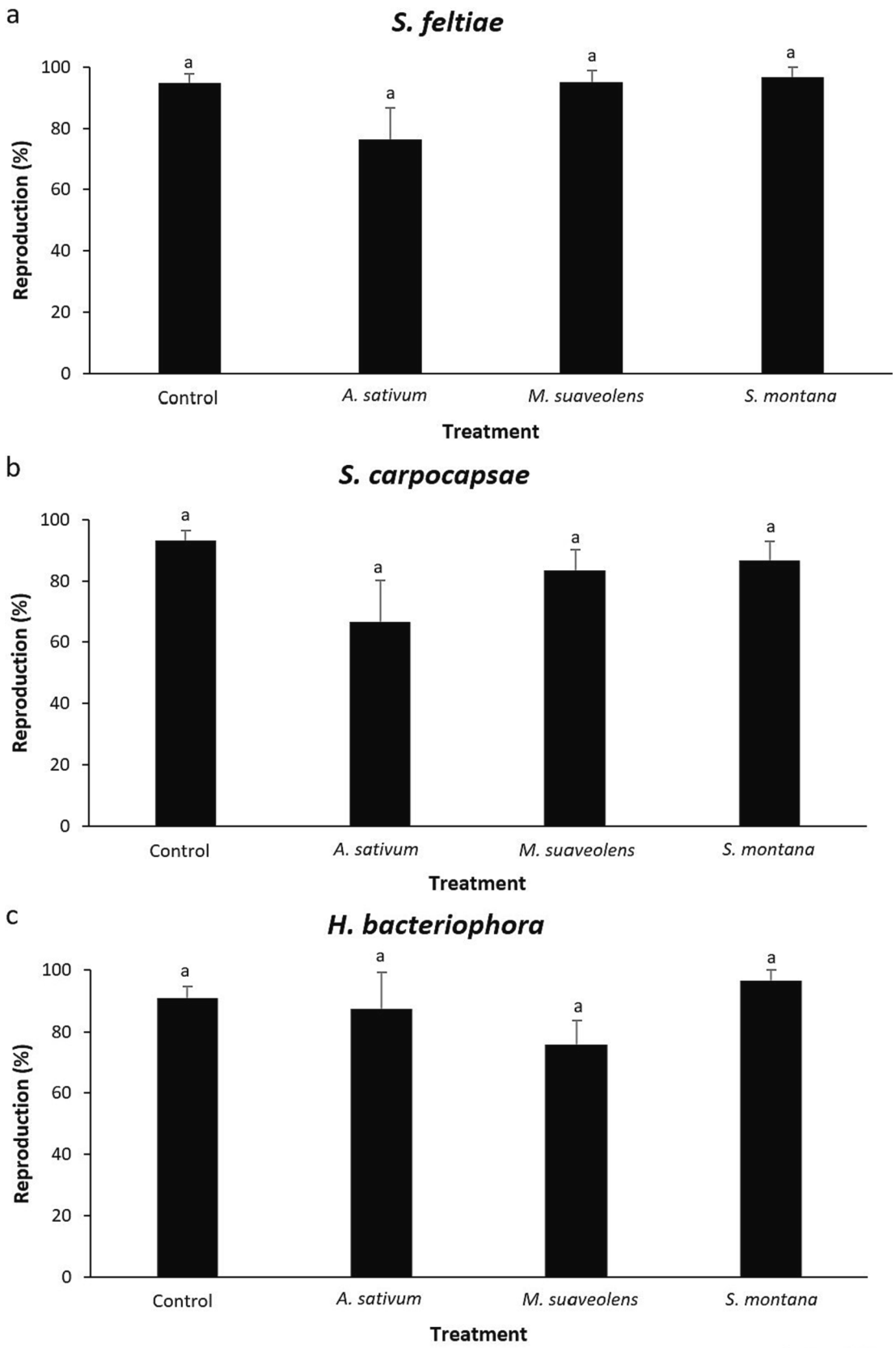

Fig. 4. Percentage of *G. mellonella* larvae (±SE) infected by surviving EPNs that reproduced within the host following sublethal exposure to EO treatments and the control. (a*) S. feltiae* (b) *S. carpocapsae* (c) *H. bacteriophora*. Within each column, different letters indicate significant differences among treatments ($p < 0.05$).

Allium sativum EO was the only treatment that caused significantly higher mortality of IJs compared to the control and other treatments at 72 h post application for *S. feltiae* (42.23 %), *S. carpocapsae* (40.81 %) and *H. bacteriophora* (40.12 %) ($F = 313.29$, $df = 3$, $p < 0.001$; $F = 236.64$, $df = 3$, $p < 0.001$ and $F = 324.15$, $df = 3$, $p < 0.001$, respectively) (Fig. 5a, 5b and 5c, respectively).

Paralysis of live nematodes was caused only by the *A. sativum* EO treatment. At 24 h, limited movement was observed, requiring light contact for nematodes to regain mobility. This paralysis became more pronounced at 72 h, demanding increased contact for movement.

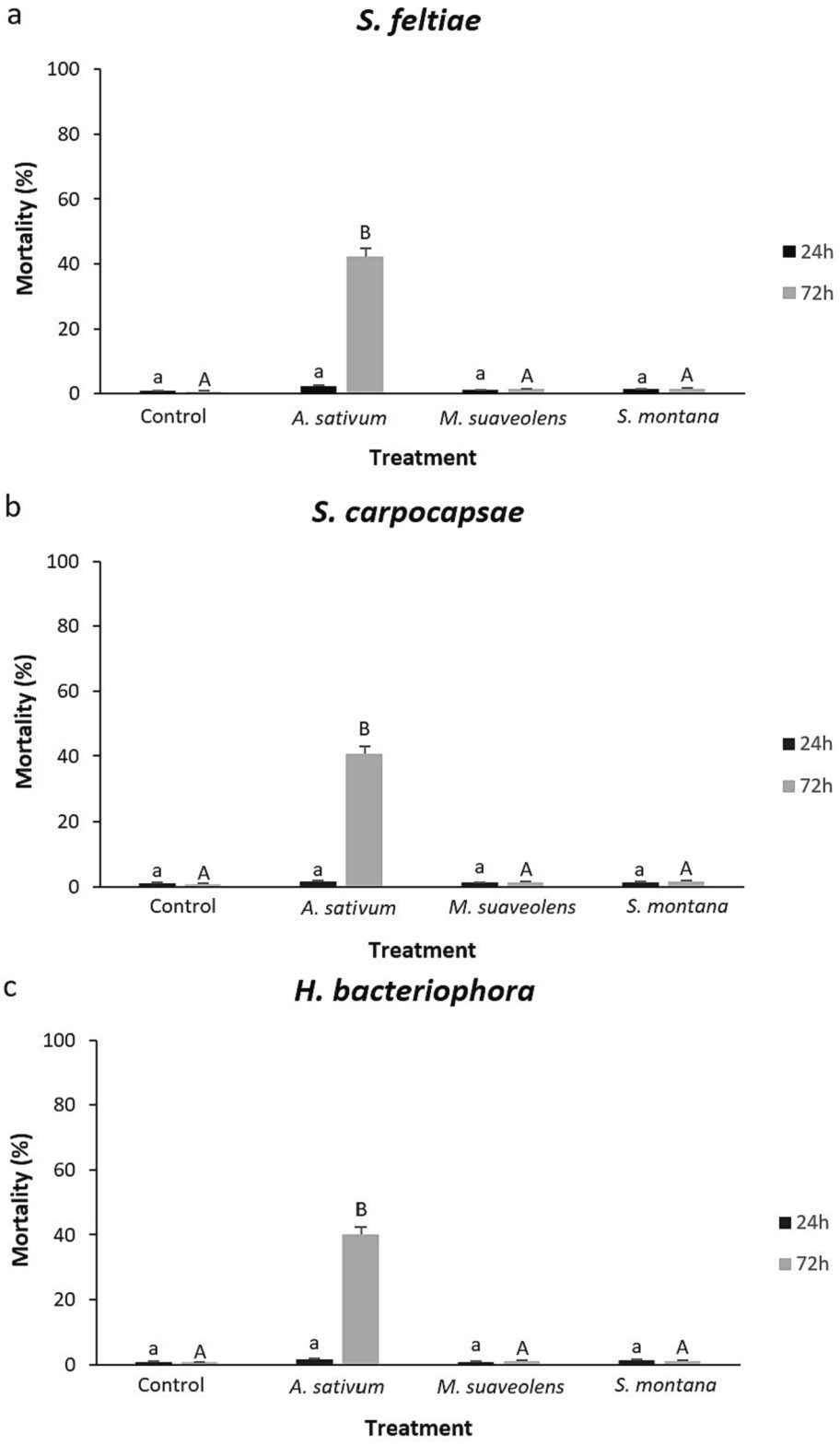

Fig. 5. Percentage of mortality (±SE) of EPNs by fumigation of three different EOs treatments and the control at 24 and 72 h post application (a*) S. feltiae* (b*) S. carpocapsae* (c*) H. bacteriophora*. Within each column, different letters indicate significant differences among treatments ($p < 0.05$).

3.3. Chemotaxis assay

The interaction between EPN species and treatment factors on the Chemotaxis Index (CI) of nematodes was statistically significant ($F = 3.62$, $df = 6$, $p < 0.01$). However, when the main effects were analyzed independently, the EPN species did not have a significant impact on CI among treatments ($F = 2.22$, $df = 2$, $p = 0.11$), while the effect of treatments was statistically significant ($F = 14.25$, $df = 3$, $p < 0.001$).

Satureja montana EO was the only treatment that significantly repelled more IJs of *S. feltiae* than the control treatment, exhibiting a stronger repellent effect compared to the other two EOs ($F = 12.66$, $df = 3$, $p < 0.001$) (Fig. 6). Similarly, *H. bacteriophora* was also more repelled by *S. montana* EO than by the control ($F = 5.74$, $df = 3$, $p < 0.01$). In contrast, *S. carpocapsae* IJs were not significantly influenced by any of the EOs ($F = 0.64$, $df = 3$, $p = 0.59$) (Fig. 6).

4. Discussion

Our results revealed that the EO extracted from *A. sativum* exhibited the highest mortality rates of *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* in the direct contact assay at both 24 h (36–38 %) and 72 h (97–99 %) post-application. Notably, *A. sativum* EO was significantly more virulent than the control and the other EOs tested. Treatments with *S. montana* and *M. suaveolens* EOs caused low mortality levels of EPNs. However, at 72 h post application, *S. montana* EO exhibited significantly more virulence (6–8 % mortality) than the control (0–3 % mortality) against all three EPN species. Few other studies have investigated the effect of EOs on EPNs. Barua et al. (Barua et al., 2020) observed that *A. sativum* and other EOs, including various *Mentha spp* EOs, caused high mortality rates for *H. bacteriophora* and *S. carpocapsae* four days post application, although *A. sativum* was significantly more toxic (100 % mortality) than *Mentha spp* (75–80 %).

The three EOs tested in the present study (*A. sativum, M. suaveolens*, and *S. montana*) are known to possess insecticidal and nematicidal properties due to their neurotoxic effects on different insects (Navarro-Rocha et al., 2020); (Valcárcel et al., 2021) and plant-parasitic nematodes (Andrés et al., 2012); (Navarro-Rocha et al., 2020) ; (Galisteo et al., 2022) . *Steinernema* and *Heterorhabditis* species share many similar basic biological and physiological characteristics with plant-parasitic nematodes, and even occupy similar soil niches (Kenney and Eleftherianos, 2016). However, in contrast to the low mortality rates caused by *S. montana* and *M. suaveolens* EOs to EPNs in our study, Andrés et al. (Andrés et al., 2012) reported that both EOs induced 100 % mortality in the J2 stage of *Meloidogyne javanica* Treub (Tylenchida: Heteroderidae) under the same concentration and exposure time. These differences between EPNs and plant-parasitic nematodes may be attributed to the cuticle. All nematodes present a cuticle rich in lipids that are soluble in substances like oils, suggesting that EOs can penetrate this cuticle and disrupt physiological processes (Page and Johnstone, 2007); (Stadler and Buteler, 2009) . However, EPN IJs have a double cuticle that most plant-parasitic nematodes do not have, which may provide them with more protection against these EOs. In fact, previous studies using chemical pesticides may have confirmed the importance of the double cuticle. They reported that IJs of *S. carpocapsae* and *H. bacteriophora* were less susceptible to various nematicides and insecticides compared to the J2 stage of *M. incognita* (Ishibashi and Ishibashi, 1985); (Ishibashi and Takii, 1993) ; (Touray et al., 2021) .

In our study, the differences in the nematicidal effect of *M. suaveolens* and *S. montana* EOs when compared to *A. sativum* may be attributed to the main compounds present in each of these oils. *Allium sativum* EO is primarily composed of organosulfur compounds, such as diallyl disulfide and diallyl trisulfide (Galisteo et al., 2022), which are inhibitors of the acetylcholinesterase enzyme (Plata-Rueda et al., 2017). This enzyme plays a crucial role in nerve transmission, and its inhibition disrupts the normal functioning of the nervous system. In contrast, the main constituents of *M. suaveolens* and *S. montana* EOs are monoterpenes that also have an impact on acetylcholinesterase (Andrés et al., 2012); (Navarro-Rocha et al., 2020) . This neurotoxic effect at the nerve transmission level is evidenced in our study by the paralysis of the nematodes after the exposure to *A. sativum*; *M. suaveolens* and *S. montana* oils at 24 and 72 h. In agreement with our results, Barua et al. (Barua et al., 2020) quantified the movement of EPN IJs exposed to different EOs, including *A. sativum* and *Mentha* spp, for 60 min and observed that IJs reduced their movements in all treatments. However, the precise mechanism by which EOs and their constituent compounds exert their nematicidal effects remains unclear and requires further investigation.

Regarding the anti-cholinesterase effect of EOs, the mechanism of neurotoxic action and symptoms are similar to those induced by organophosphates and carbamates insecticides. Numerous studies have investigated the effects of these insecticides on EPNs. Gordon et al.

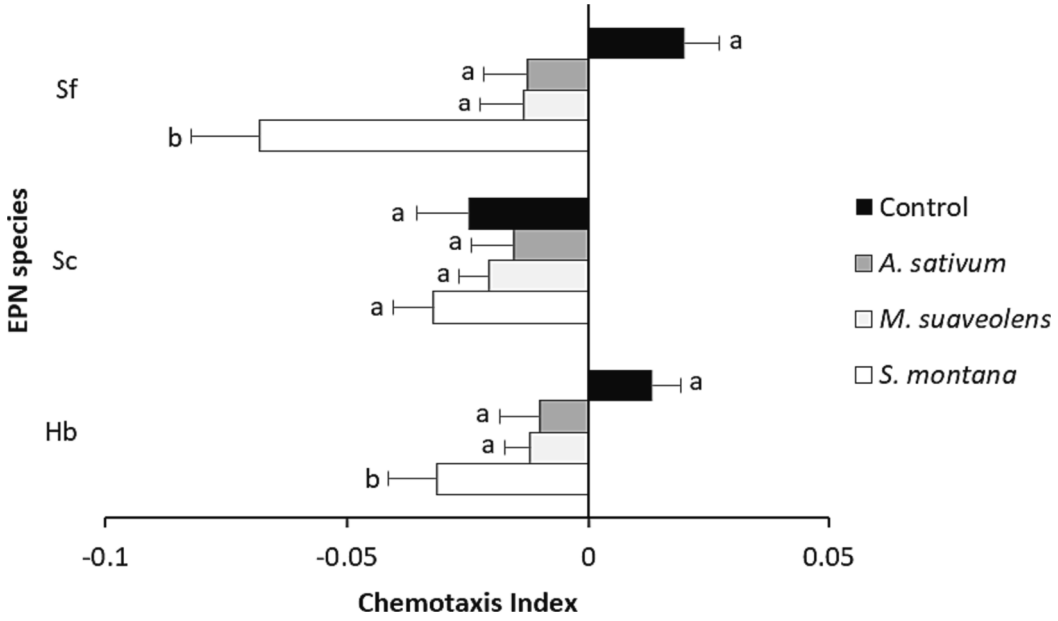

Fig. 6. Effect of different EOs on the chemotactic response (CI ± SE) of EPNs at 4 h post application. Different letters indicate significant differences among EOs and the control for each EPN species ($P < 0.05$). Sf: *Steinernema feltiae*, Sc: *Steinernema carpocapsae*, Hb: *Heterorhabditis bacteriophora*.

(Gordon et al., 1996) studied the effect of two carbamates, carbofuran and fenoxycarb, on the survival of infective juveniles (IJs) of *S. feltiae* and *S. carpocapsae*. The results indicated that both nematode species experienced mortality when exposed to these insecticides. In a study by Gaugler and Campbell (Gaugler and Campbell, 1991); *H. bacteriophora* was subjected to oxamyl, a carbamate, revealing that the compound caused aberrant nematode movement and partial paralysis due to its neurotoxic effect, obtained from the partial carboxylation of acetylcholinesterase. Similar results were obtained with organophosphate insecticides. Amizadeh et al. (Amizadeh et al., 2019) reported early toxicity in *S. feltiae* after application of certain organophosphorus compounds. Antagonistic effects were also observed when EPNs were applied immediately or 12 h after the insecticide treatments due to acetylcholinesterase inhibition. Similar experiments on *S. carpocapsae* yielded analogous results (Ishibashi and Takii, 1993). Therefore, when comparing these studies with our results, it is suggested that *A. sativum* EO may exert toxicity on EPNs comparable to that of organophosphate and carbamate insecticides, indicating a similar mode of action.

The sublethal effect assay showed that *A. sativum* EO was the only oil affecting the infectivity of all three EPN species. The high neurotoxic effect observed with *A. sativum* EO in our study, as also noted by Barua et al. (Barua et al., 2020); likely underlies its persistence effect over the long term, even after the nematode exposure to this oil was ended. This caused them to lose their infective capacity, either due to impaired movement or direct mortality. In contrast, in our study, treatments with *M. suaveolens* and *S. montana* EOs did not affect the infectivity of EPNs. Survival curves of *G. mellonella* from *M. suaveolens* and *S. montana* treatments were not significantly different from the control, suggesting that the previously observed paralysis of IJs did not affect their ability to infect *G. mellonella* larvae. Furthermore, none of the EO treatments exhibited any significant effect on the reproduction of EPNs infecting *G. mellonella*.

EOs can act as fumigants on insects and nematodes due to their high volatility and active compounds (Shaaya et al., 1991); (Laquale et al., 2015) . Although there are no studies on the fumigant effect of EOs on EPNs, research on plant-parasitic nematodes has demonstrated high mortality rates. Laquale et al (Laquale et al., 2015) reported reduced multiplication of *M. incognita* after exposing tomato plants to various EOs for two months in a greenhouse at 25 °C. In our study, the results of the fumigation assay were consistent with those of the direct contact assay, showing that *A. sativum* EO was significantly more lethal to EPNs than the control, *M. suaveolens*, and *S. montana* EOs. However, the mortality of EPNs exposed to *A. sativum* EO reached approximately 40 % 72 h post application, which is significantly lower than the mortality observed in the direct contact assay (97–99 %). These differences are also evident when observing the mobility of nematodes at 24 and 72 h post application. In contrast to the direct contact assay, no paralysis of the nematodes was caused by *M. suaveolens* and *S. montana* EOs in the fumigation assay. Therefore, the different ways of exposure to EOs may explain these results.

The chemotaxis assay conducted in our study showed that *S. montana* EO induced a significant repellent behavior in *S. feltiae* and *H. bacteriohora* compared to the control. In contrast, *A. sativum* and *M. suaveolens* EOs did not show significant effects, neither attraction nor repulsion, on EPN behavior. To date, this study represents the first evidence of the repellent effect of EOs on EPNs, despite existing studies demonstrating this effect on insects, including *A. sativum* and *M. suaveolens* EOs (Mann et al., 2011); (Manh and Tuyet, 2020) . The repellent effect of EOs is likely a result of the combined impact of all their compounds. In the case of *Satureja* spp oils, the main compounds usually include the monoterpenes thymol and carvacrol (Taban et al., 2017). Taban et al. (Taban et al., 2017) observed strong repellent activity against the beetle *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) across the EOs extracted from three *S. montana* species. Moreover, Tabari et al. (Tabari et al., 2017) noted that thymol and carvacrol were highly repellent to the tick *Ixodes ricinus* (L., 1758) (Acari: Ixodidae). In our study, the fact that EPNs were not repelled by the highly nematicidal *A. sativum* oil suggests that these nematodes may lack avoidance behavior towards some EOs, even though these products can be toxic to them. Ulu (Ulu, 2023) similarly observed a lack of avoidance behavior in EPNs when exposed to certain chemical pesticides. Therefore, our results underscore the importance of considering the repellent effect of EOs when using them in conjunction with EPNs.

Overall, our study demonstrates that *M. suaveolens* EO may be the most compatible oil to use in conjunction with EPNs to control the beetle *L. cinnamomeus* in truffle plantations. Although *S. montana* EO also caused low mortality rates of EPNs at 72 h post application and did not affect their infectivity, it was repellent towards *S. feltiae* and *H. bacteriophora*, so their use in conjunction should be evaluated. In contrast, applying *A. sativum* together with *S. feltiae*, *S. carpocapsae*, and *H. bacteriophora* at the same time is not recommended due to the lethal and sublethal effects on EPNs directly exposed to this EO. Furthermore, the application of *A. sativum* EO and EPNs at different times should not be recommended, considering the results obtained in the fumigation assay. Therefore, it would be interesting to study the residual effects of EOs over time on EPNs to predict a pattern for the application of these two control agents in the field.

5. Conclusions

To conclude, our results demonstrate that *A. sativum* EO caused high mortality rates in *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* at 24 and 72 h post application whether by direct contact (97–99 %) or fumigation (40–42 %), and it also affected their infective capacity. *S. montana* EO caused low mortality rates (6–8 %) at 72 h in the lethal assay, displaying repellent properties against *S. feltiae* and *H. bacteriohora*. In contrast, *M. suaveolens* EO demonstrated a lack of significant impact on the survival, infectivity and reproduction of the three EPN species tested. Therefore, our results suggest *M. suaveolens* oil may be the most compatible EO to use in conjunction with EPNs against *L. cinnamomeus* in the field. We also emphasize the importance of careful consideration when incorporating EOs into integrated pest management (IPM) strategies alongside EPNs. However, it is crucial to note that these laboratory results need validation under field conditions and in the presence of *L. cinnamomeus* to ascertain their practical applicability. Furthermore, further studies should also evaluate the effects of other EOs on EPNs to expand our understanding of these interactions. This comprehensive approach will contribute to a more informed and effective utilization of EPNs in conjunction with EOs in sustainable pest control strategies.

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CRediT authorship contribution statement

Ivan Julià: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Marina Seco de Herrera:** Methodology. **Ana Morton:** Writing – review & editing, Investigation. **Anna Garriga:** Writing – review & editing, Investigation. **Daniel Tapia:** . **Juliana Navarro-Rocha:** Methodology. **Fernando Garcia-del-Pino:** Writing – review & editing, Validation, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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