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Nematicidal Effects of Four Terpenes Differ among Entomopathogenic Nematode Species

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Abstract: Entomopathogenic nematodes (EPNs) have been studied for more than half a century, and employed for insect pest management using augmentation, conservation, and classical biological control approaches. As obligate lethal parasitoids of insect larvae, EPN navigate a chemically complex soil environment and interact with their insect hosts, plants, and each other. EPN responses to various terpenes, such as herbivore-induced plant volatiles, have the potential to enhance EPN efficacy through their attraction. However, several of the terpenes are currently being formulated as biological fungicides, insecticides, and acaricides for above- or below-ground applications. We conducted laboratory experiments to investigate the possible nematicidal effect of four terpenes, carvacrol, geraniol, eugenol, and thymol, to two heterorhabditids and two steinernematid species. Each terpene showed nematicidal activity against at least two of the four EPN species, with carvacrol showing the strongest activity and *Heterorhabditis bacteriophora* the highest sensitivity. Despite the high sensitivity of both heterorhabditids and near-zero sensitivity of the steinernematids to thymol, carvacrol, and eugenol, an increasing effect was observed when steinermatid nematodes were exposed to geraniol, and a decreasing effect for heterorhabditids, with *H. bacteriophora* exhibiting higher mortality than *H. indica*. The virulence of the nematodes towards fourth instar *Galleria mellonella* was also tested after exposure to the median lethal doses of each terpene. No significant difference in virulence was observed between nematodes that were exposed or not exposed to sublethal doses. The experiments suggest that the tested terpenes have a strong effect on EPN viability, which should be considered when combining the two approaches in IPM. The terpenes did not have a universal effect on all nematode species, which merits further investigation, while virulence tests suggest that sublethal doses of these terpenes have no effect on the host-killing performance of EPNs.

Keywords: entomopathogenic nematodes; terpenes; nematicidal activity; eugenol; carvacrol; thymol; carvacrol; nematostatic activity

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1. Introduction

Entomopathogenic nematodes (EPN; genera *Steinernema* and *Heterorhabditis*) are a group of nematodes that have the ability to infect and kill insect pests. These pathogens have been widely studied and used as biological control agents against a wide range of economically important insects [1–3]. These nematodes have a symbiotic relationship with bacteria (*Xenorhabdus* spp. and *Photorhabdus* spp. for steinernematids and heterorhabditids, respectively), with which they induce a complex insecticidal effect to their host. Infective juveniles (IJs), the only free-living stage, can enter their hosts through natural openings (mouth, anus, and spiracles) or, in some cases, through the cuticle [4]. After penetrating the host's hemocoel, the nematodes molt and complete up to three generations before exiting in search of new hosts [2,5]. Once they enter the insect body, the nematodes release toxins and spread their symbiotic bacteria killing the host within 24–48 h [6]. The bacteria consume the insect hemolymph as a source of nutrition and inundate the insect cadaver while releasing

a range of bioactive chemicals with insecticidal activity [7]. In this mutualistic relationship, the nematodes protect the bacteria from harsh conditions outside their insect host, while the bacteria repel other invaders and provide nutrients to the nematode partners [2,4].

Terpenes are a large and diverse class of natural compounds that are produced by many plant species, and they play a crucial role in the defense mechanisms of plants against herbivores, pathogens, and other stress factors [8–10]. They exhibit a broad spectrum of effects, ranging from toxicity to insects [11], fungi [12], and bacteria [13], to serving as feeding deterrents to mollusks [14], insects [15], and mammals [16]. Due to their natural origin and low toxicity to humans and the environment, terpenes have emerged as promising alternatives to synthetic pesticides in plant protection strategies, with numerous commercial products developed for pest control. Terpenes, being a primary element of essential oils, have been employed in traditional agricultural methods for centuries to combat pests, and are recognized as environmentally safe [17–19]. Terpenes such as thymol, carvacrol, eugenol, and geraniol are commonly found in herbs, including thyme, oregano, basil, and lemongrass. These terpenes have been found to be effective against a wide range of soil-borne diseases and insect, nematode, and mite pests [20]. They have been tested for biocontrol efficacy against a broad group of parasitic nematodes including root knot, lesion, pine wilt, and bulb nematodes [21–26]. Terpene mixtures significantly reduced the number of nematodes in the soil, suggesting that they could be used as effective natural nematicides for controlling root knot nematodes in soil [27–29].

There is also a plethora of studies focusing on terpenes as allomones in nematode ecology particularly as a type of semiochemicals produced and released by plants that affect the behavior of nematodes. Entomopathogenic nematodes have evolved the ability to use various plant signals to locate potential herbivore hosts. Roots infested by herbivores release a specific blend of molecules that is different from the molecules released by healthy plants. The abundance and diffusion of these herbivore-induced plant volatiles (HIPVs) is a detectable, although not reliable, indicator of herbivore presence [30–32]. Belowground observations and EPN behavior assays performed in Pluronic gel showed that EPN aggregated near plant roots and preferentially oriented to injured plants. HIPVs from various plant species, including maize, citrus trees, potato, sugarcane, carrot, and grapevine, induce chemotaxis in EPNs [31,33–39]. Two broad categories of foraging strategies have been recognized for EPNs, cruisers and ambushers [40]; both are attracted to HIPVs, but the response to different volatiles is strain-specific feature rather than associated with EPN foraging strategy [41].

Since terpenes are compounds used in plant protection products and are compatible with organic agriculture in many countries, it is important to understand the effect of these compounds in combination with other EPN approaches such as conservational or augmentative biological control. Here we evaluate the nematicidal effect of four terpenes, thymol, carvacrol, eugenol, and geraniol, on the EPNs, *Steinernema feltiae* (Filipjev, 1934), *Steinernema carpocapsae* (Weiser, 1955), *Heterorhabditis bacteriophora* (Poinar, 1976), and *Heterorhabditis indica* (Poinar, Karunakar & David, 1992) and the ability of these nematodes to kill their host after exposure to sublethal doses.

2. Materials and Methods

2.1. Survival Bioassays

The nematicidal effect of four terpenes, thymol, carvacrol, geraniol, and eugenol, were tested on infected juveniles of four EPN species, *S. feltiae*, *S. carpocapsae*, *H. indica*, and *H. bacteriophora*. The IJs of EPNs were exposed to different concentrations (500, 1000, 1500, and 2000 ppm) of tested terpenes, and the survival of IJs was evaluated 24, 48, and 72 h after treatment. The experiment was carried out in sterile 24-well polystyrene plates, using five wells for each species and one separate plate for each terpene, including the control (IJs without exposure to any terpene) in distilled water. Each experimental unit (a well containing around 50 IJs) was exposed to one of four concentrations of a terpene and had 6 replicates (24 wells in total). The motility of infective juveniles was assessed every 24 h by

disturbing them with a fine brush. Nematodes that showed no movement were classified as dead. To prevent moisture loss and terpene evaporation, each plate was sealed with parafilm and covered with plastic lids. The plates were opened and inspected at 24, 48, and 72 h, then sealed again. The experiment was repeated on a separate date, and the data from both experiments were combined and analyzed together since no differences were found.

2.1.1. Source and Preparation of Nematodes

The EPN cultures were maintained on larvae of *Galleria mellonella* L. (Lepidoptera, Pyralidae) separately under laboratory conditions (25 °C, 50% RH). The population of *H. indica* was originally extracted from citrus fields in Florida, USA, and obtained from the nematology laboratory of the Citrus Research and Education Center at the University of Florida, while the other three EPN species were originally obtained from commercial products: *S. feltiae* (nemaplus®, E-nema Corporation), *S. carpocapsae* (nemastar®, E-nema Corporation), and *H. bacteriophora* (nematop®, E-nema Corporation). When EPNs were received by the lab, their identification was confirmed by the amplification of the internal transcribed spacer region using the primer set TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and AB28 (5′ATATGCTTAAGTTCAGCGGGT-3′), while Sanger's sequencing data including the forward and reverse DNA strands, were manually assembled and edited, constructing a neighbor-joining tree using MEGA (version 11.0.13). All the sequences obtained have been submitted to NCBI GenBank under the accession number OQ998912 for *H. bacteriophora*, OQ998935 for *S. feltiae*, OQ998942 for *H. indica*, and OQ998952 for *S. carpocapsae*.

All strains were reared in the last instar of *G. mellonella* at 25 °C. Infective juveniles were harvested as aqueous suspensions (in distilled water), 3 to 5 d after their first emergence from the cadaver. Prior to testing, IJs were washed with distilled water and placed in a Baermann funnel for 2 h. After collection, their viability was determined by microscopic examination. The populations that exhibited viability of between 95 and 100% were included in the bioassays. The IJs were put into each well at the concentration of 100 IJs/mL water, and the final number of IJs in a well was around 50 individuals.

2.1.2. Preparation of Terpene Solutions

The terpenes were commercial products bought from corporations certifying higher than 97% purity (see supplementary files). Terpenes was dissolved in high purity ethanol (>98%). All terpenes, except thymol, were in liquid form, so for their dissolution, 160 µL of terpene oil was dissolved in 800 µL ethanol. In the case of thymol in solid form, 0.16 g was weighed and dissolved in 800 µL of ethanol. The prepared solutions were diluted in water with tween 20 at a concentration of 0.6% to a volume of 40 mL in 50 mL. Falcon tubes to obtain the stock solutions, of terpenes at concentrations of 4000 ppm. For each impending test, working solutions were prepared at twice the concentration, and the well was filled with 0.5 mL of working solution and 0.5 mL of nematode suspension.

2.2. Pathogenicity Bioassays

This test was performed to determine the negative effect of sublethal doses of terpenes on the pathogenicity of IJs. For this purpose, a concentration close to the LC_{50} was chosen for each terpene. The pathogenicity of IJs that were exposed to LC_{50} values of each terpene was tested against the last instar of *G. mellonella* and mortality of the larvae was recorded daily for 3 d after application. The bioassays were conducted in Petri dishes (55 mm diameter) filled with 8 cm^3 of sand adjusted to 12% humidity (*w*/*w*) and containing 5 to 20 IJs (depending on species) and a single last-stage waxworm *Galleria mellonella* larva.

Prior to bioassays, IJs were washed with distilled water and placed in a Baermann funnel for two hours. The collected individuals were tested for their viability and concentrated in aqueous suspension of 100 IJs/mL. Five mL of nematode suspension (500 IJs) was transferred into a glass Petri dish (12 cm diameter) and 5 mL of the terpene was added. Then, the plates were lightly shaken, sealed with parafilm and incubated at 20 °C for 24 h. After incubation, nematodes were evaluated for mortality. To maintain consistent mortality levels

with those seen in the control group while also keeping them under 100%, adjustments had to be made regarding the number of nematode inoculations used. Nematodes were harvested in deionized water, from which they were individually picked up with a pipette and transferred to arenas (5 IJs for *S. feltiae* and *S. carpocapsae*, 10 IJs for *H. bacteriophora*, and 20 IJs for *H. indica*). To avoid loss of IJs on the inner pipette surfaces, the tips were first washed in 2% Triton X-100. Finally, the arenas were placed in boxes in groups of five and kept at 25 °C until the end of the experiment. Every 24 h, larvae were checked to see if they were still alive by opening each arena and probing the larvae with forceps.

2.3. Statistical Analysis

The first experiment had a full factorial design through a repeated measures model (MANOVA), where the response variable was nematode mortality ratio in a well. To acquire a normal variance, prior to the analysis, data were converted to log (x + 1). The repeated factor was the exposure time of the nematodes to the terpene (24, 48, and 72 h) and the main effects were nematode species, terpene, and terpene dose/concentration. Means were discriminated with the Tukey–Kramer HSD test (comparing all pairs) at the 5% significance level. Statistical analysis was performed using JMP Pro, v16.2 (SAS Institute Inc., Cary, NC, USA). Before statistical analysis, all numbers were calculated according to Abbott's formula [42]:

$$\propto = \frac{mortality\ treatment - mortality\ water}{100 - mortality\ water} \times 100\%.$$

Each experimental unit had six replicates and the entire mortality bioassays were repeated using new EPNs and freshly prepared terpenes at all concentrations (2 experiment repeats × 6 replications wells, 50–60 individuals per well × 4 EPN species × 4 terpenes × 4 doses/control).

In the second experiment, a Kruskal–Wallis test was used to evaluate the significance of differences in the mean mortality of *G. mellonella* by nematodes exposed to the four terpenes and the control (n = 8).

3. Results

All main effects and their interactions (nematode species, terpene, terpene concentration, and exposure time) were significant in relation to nematode mortality. Nematode species was the most important factor affecting nematode mortality, as it explained the largest part of variability in the data, followed by terpene concentration, and the interaction between nematode species and the terpene compound (Table 1).

Table 1. MANOVA parameters for main effects and associated interactions for mortality of entomopathogenic nematode infective juveniles between and within exposure intervals (error DF = 702).

Effect	**Mortality**		
Source	**DF**	***F***	***p***
Between exposure intervals			
Intercept	1	92,096.90	<0.0001
Nematode species	3	9280.10	<0.0001
Terpene	3	100.3	<0.0001
Terpene dose	3	1389.30	<0.0001
Nematode species × Terpene	9	1388.90	<0.0001
Nematode species × Terpene dose	9	54.7	<0.0001
Terpene × Terpene dose	9	109.5	<0.0001
Nematode species × Terpene × Terpene dose	27	50.5	<0.0001
Within exposure intervals			

Table 1. *Cont.*

Effect	Mortality		
Source	**DF**	***F***	***p***
Exposure	2	338.7	<0.0001
Exposure × Nematode species	6	34.5	<0.0001
Exposure × Terpene	6	6.8	<0.0001
Exposure × Terpene dose	6	28.1	<0.0001
Exposure × Nematode species × Terpene	18	17.3	<0.0001
Exposure × Nematode species × Terpene dose	18	26.9	<0.0001
Exposure × Terpene × Terpene dose	18	17.4	<0.0001
Exposure × Nematode species × Terpene × Terpene dose	54	13.1	<0.0001

The effects of the four terpenes on the mortality of four species of EPN IJs is shown in Figure 1. The percentage of dead nematodes increased with increasing concentration and exposure time (Figure 1). Heterorhabditids showed higher sensitivity to the terpenes than steinernematids, with *H. bacteriophora* showing more than fourfold mortality compared to *S. carpocapsae* and threefold mortality compared to *S. carpocapsae*. Carvacrol proved to be the most toxic of the terpenes, but only carvacrol and thymol were found to be significantly higher than eugenol with the lowest effect.

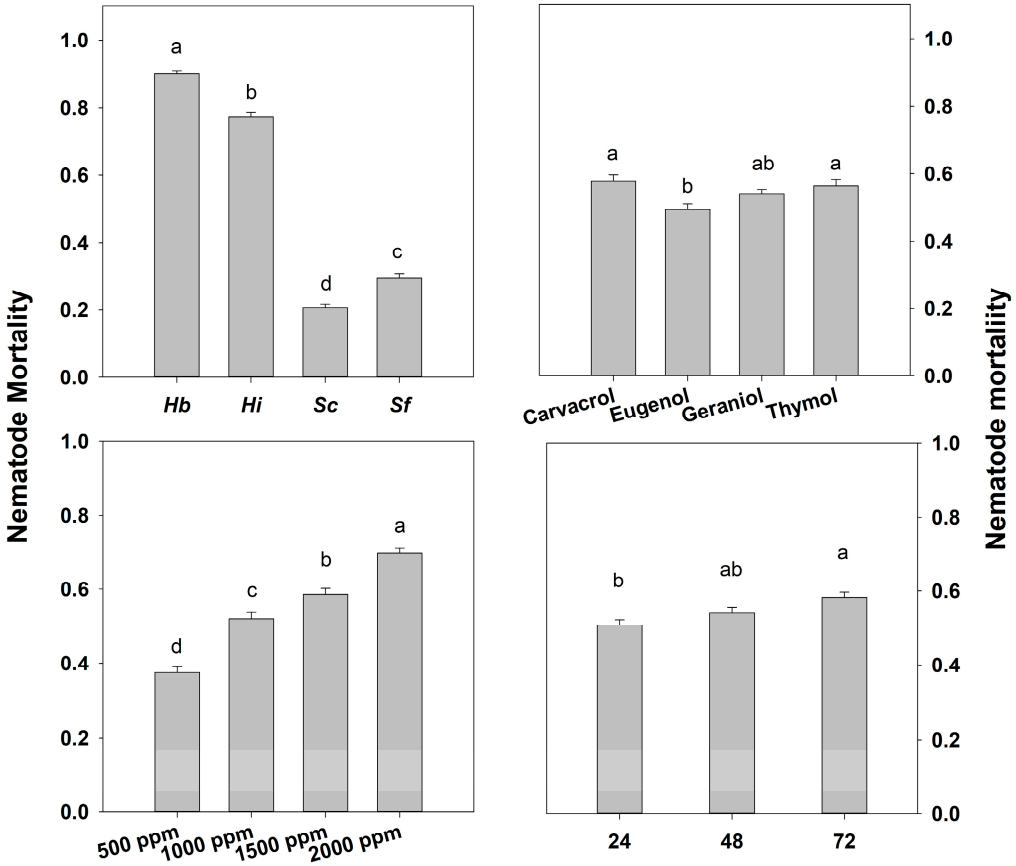

Figure 1. Mortality ratio (0–1) of infective juveniles affected by the main key factors when four different entomopathogenic nematode species (upper left: Hb, *Heterorhabditis bacteriophora*; Hi, *Heterorhbditis indica*; Sc, *Steinernema carpocapsae*; Sf, *Steinernema feltiae*) exposed to four different terpenoids (upper right) in four different concentrations (lower left) for three days (lower right: 24-, 48- and 72-h exposure). Error bars are SE of the means, different letters above bars indicate statistical significance ($p < 0.05$).

Both heterorhabditid species exhibited high mortality, of up to 100%, when exposed to thymol, carvacrol, and eugenol (Figure 2). In the carvacrol and thymol treatments, heterorhabditids reached 100% mortality at the lowest concentration with *H. indica* being slightly less responsive, requiring 48 h exposure to reach complete mortality. Eugenol was also highly toxic for the heterorhabditids, achieving 100% mortality at 1000 ppm and killing 40% (Hi) and 53% (Hb) at 500 ppm at the first 24 h. *Heterorhabditis indica* was the least susceptible EPN species, responding to geraniol with 6% mortality at the lowest concentration (500 ppm), and exhibiting 55% after 72 h. Ninety six percent of *H. bacteriophora*, more than any other species, were killed by geraniol.

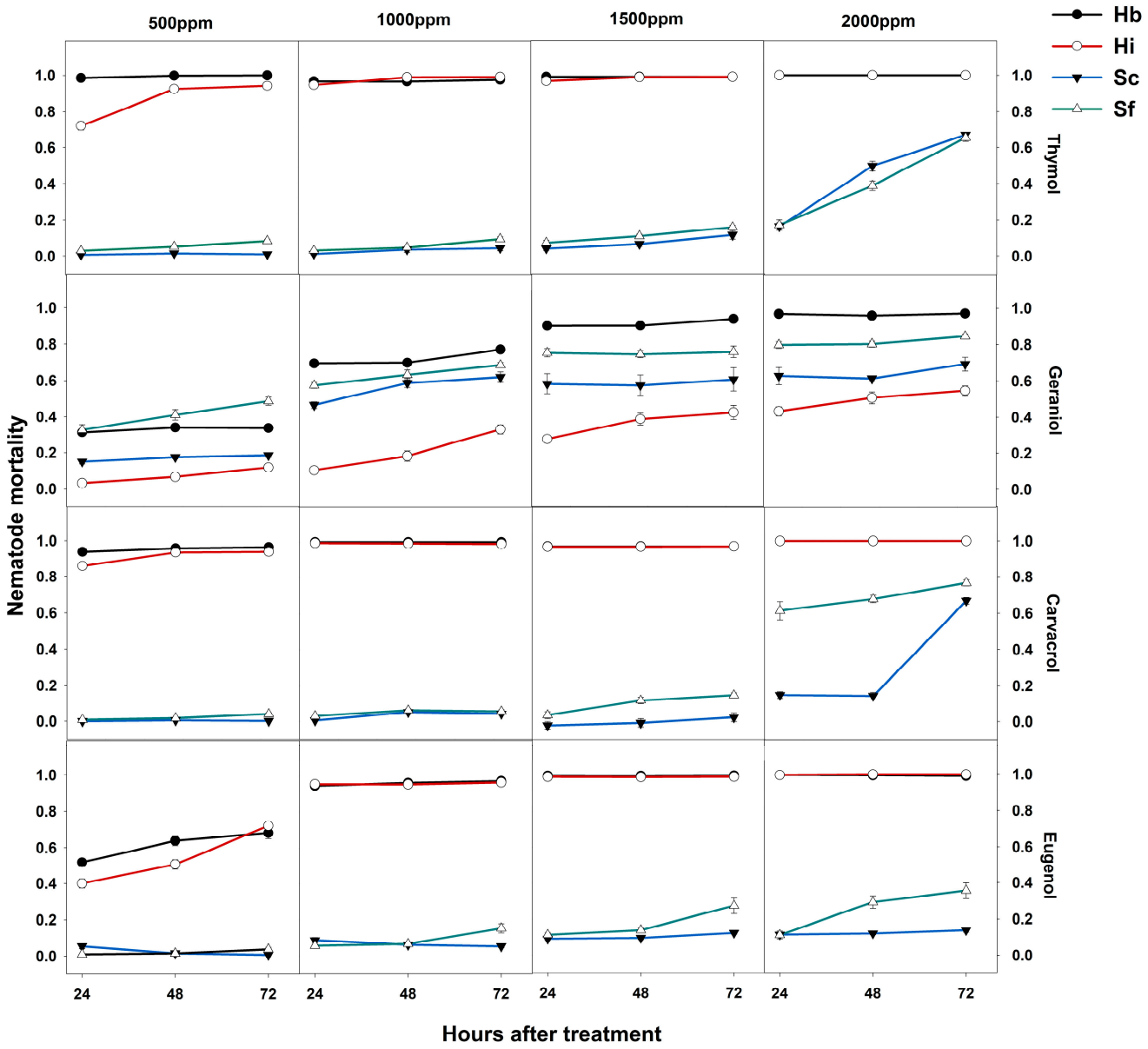

Figure 2. Infective juvenile mortality ratios (0–1) of four different entomopathogenic nematode species (Hb, *Heterorhabditis bacteriophora*; Hi, *Heterorhbditis indica*; Sc, *Steinernema carpocapsae*; Sf, *Steinernema feltiae*) exposed to four different terpenoids (rows; thymol, geraniol, carvacrol, eugenol) in four different concentrations (columns; 500, 1000, 1500, and 2000 ppm) for three days (24, 48, and 72 h of exposure). Error bars are SE of the means.

By contrast, both steinernematids were affected mainly by geraniol. The lowest dose killed up to 18.5% (Sc) and 48.5% (Sf), with 69.2% of *S. carpocapae* and 82.8% of *S. feltiae*

dying at the highest concentration of 2000 ppm. The toxic effects of thymol and carvacrol against steinernematids became evident only at 2000 ppm, increasing to 66.8% (Sc) and 41.2% (Sf) for thymol, while mortality increased up to 66.8% (Sc) and 76.6% (Sf) for carvacrol after 72 h of exposure. Eugenol was found to have a significant effect on *S. fetiae*, but it did not exceed 35.7% mortality, while it had a non-significant effect on *S. carpocapsae*. All mean values of nematode responses to the terpene, species, dose, and time effect are shown in the supplementary files, providing further information on mean comparisons.

In the second experiment to evaluate nematode performance after exposure to the sublethal doses detected in the first experiment, no differences were detected among the treatments ($p > 0.05$). No mortality was observed 24 h after treatment. Mortality increased after 48 (Figure 3) and 72 h of exposure to the nematodes, but in neither case did any of the terpenoids significantly decrease or increase nematode performance.

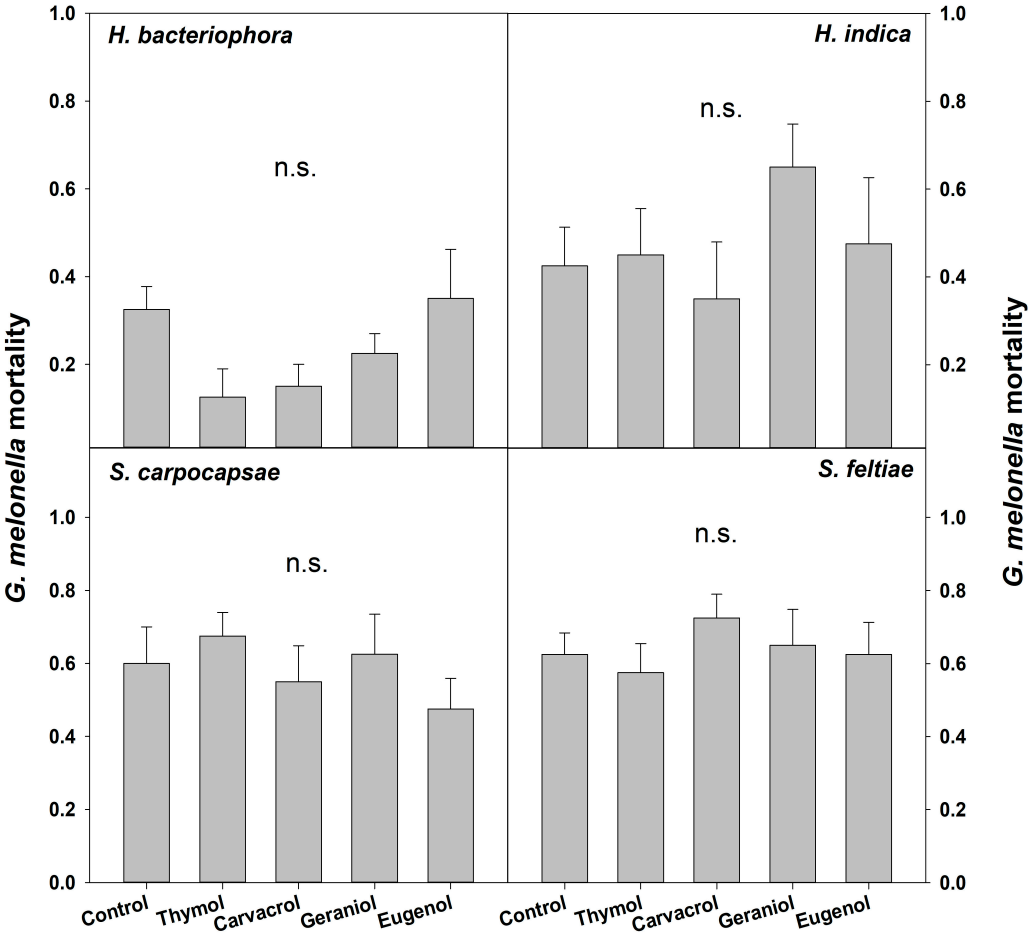

Figure 3. *Galleria mellonella* mortality ratio (0–1) after a 48 h exposure to four entomopathogenic nematode species pre-exposed to four terpenoids and water (control) in sublethal doses for 24 h. Error bars are SE of the means, with "n.s." indicating non-significance within the group.

4. Discussion

Terpenoids were found to be deleterious to the EPN species tested, but with different results for mortality among the four species. Thymol, carvacrol, and eugenol were found to be highly toxic to heterorhabditid species, but only to steinernematids in very high amounts or long exposures. By contrast, geraniol was found to be toxic to steinernematids, but with less effect on heterorhabditids. Ours are not the only examples of species-specific responses to terpenes for nematodes. There are reports indicating different degrees of toxicity of terpenoids for a long list of nematodes.

The literature on the nematicidal effects of terpenes is confusing because in most cases the material tested is not the isolated terpene but the essential oil of a plant, in a mixture with other constituents from numerous plants. Comparison of results between studies is impractical because the developmental stage tested is not always the same and the environment, habitat, and nematode host dictate a completely different regime of chemical interactions. Numerous studies have focused on anthelmintic properties of these terpenes, reporting a relatively high toxicity on animal parasitic nematodes such as *Haemonchus contortus* and *Ascaris suum* [43,44]. Considering the soil environment where both EPNs and plant parasiticic nematodes (PPNs) produce a resistant stage to navigate rhizosphere in a search for a new host, plant parasitic nematodes are another well tested group for toxicity to the terpenes. Root knot nematodes (RKNs) have shown high sensitivity, with carvacrol, geraniol, and eugenol being able to kill more than 90% of the *Meloidogyne javanica* J2 at 500 ppm, even after 24 h of exposure, according to Nasiou and Giannakou [22–24], while Abdel Rasoul [45] reported LC_{50} doses for *Meloidogyne incognita* J2s of 170 ppm and 198 ppm for geraniol and thymol, respectively. In pinewood nematodes, terpenes also exhibit high toxicity. The LC_{50} values of thymol and carvacrol against juveniles were reported to be 96 ppm and 99 ppm, followed by geraniol at 415 ppm, among 26 monoterpenoids that were bioassayed [46]. By contrast, *Ditylenchus dipsaci* was relatively insensitive to carvacrol, reaching 100% mortality only at a concentration of 2000 ppm, followed by eugenol > geraniol > thymol in descending order of toxicity [26].

Differences in family level response of EPNs could be attributed to their evolutionary distance. Although Steinernematidae and Heterorhabditidae share morphological and ecological similarities, they are not closely related, while affinities likely result from convergent evolution [47,48]. Lower survival rates for heterorhabditids than for steinernematids have been reported primarily by Gaugler [49], which could be translated to a higher susceptibility to these terpenes for the heterorhabditids. Strong [50] has extensively studied the survival of IJs in soil and found that heterorhabditids have a shorter half-life than *Steinernema*. He attributes these differences to their different strategies for overcoming stressful environmental conditions and being active when a host is available.

It is also known that terpenes (e.g., limonene, pinene, caryophyllene, and pregeijerene) are an important component of the EPN repertoire for communicating with plants and finding an available host [51]. However, not all EPN species respond equally to these compounds. A terpene may be attractive to one EPN species and repellent to another, which could explain different susceptibility levels when two species are exposed to a terpene. Further work with additional EPN species could validate a universal mechanism and shed light on the mode of action of those terpenes, such as thymol, carvacrol, and eugenol, that are poorly understood. Although, geraniol was found to have a similar effect on both steinernematids, heterorhabditids responded quite differently, indicating an intrafamily variability.

Our hypothesis, that terpenes could have an effect on nematode performance when they are exposed to sublethal doses, was not supported. Nasiou & Giannakou [23] observed a sublethal dose–effect relationship when geraniol was used to prevent RKN *Meloidogyne javanica* invasion in roots, suggesting that the duration of exposure and the concentration of the terpene could disorientate nematodes during root location. Current results show that entomopathogenic nematodes are not affected by the terpene and, if not killed, they still have the ability to find and successfully kill the target host. These findings make EPN more compatible with other pest management approaches, eliminating the possibility of an unintended effect of a terpenoid application to the soil.

The current result has implications for two main concepts. First, in the case of inundative/inoculative biological control, the current results could provide guidance on the type and dosage of a terpenoid that should be combined either in soil application or in combination with other pest management approaches. The success of biological control is influenced by numerous agricultural inputs such as fertilizers, chemical pesticides, or biological control practices, that can have positive, neutral, or negative effects on EPN. Many

chemical pesticides, such as abamectin and aldicarb, are toxic to EPNs, while others tend to be compatible, even acting synergistically in the case of carbaryl and imidacloprid [52–54]. Nevertheless, combinations should be tested on a case-by-case basis.

Indirect effects of IPM practices on native entomopathogenic nematode populations should also be considered [55–57]. As the tested terpenes exhibit specificity in killing EPNs, current results can be also a useful guide for the use of terpenes in the field, taking into account EPN conservation. This requires knowledge of EPNs' biogeography and the species present in a treated area. Advances in molecular tools have made nematode detection faster and less expensive, bringing conservational biological control of EPN populations within reach. The results may suggest alternative terpenes that reduce the risk to non-target organisms and natural enemies such as EPN when selectivity is claimed and use in integrated pest management systems is sought.

In summary, terpenoids such as thymol, carvacrol, and eugenol, used commonly for pest control, should not be combined with *H. indica* and *H. bacteriophora* or should be avoided in areas where these species are prevalent. Alternatively, they could be combined with *S. feltiae* and *S. carpocapsae*, which appear to be relatively resistant to these terpenes. Geraniol does not appear to be compatible with any EPN species. Despite the fact that, in some cases, the terpenes only reached the LC_{50} at high concentrations, all species showed significant sensitivity. Future research could focus on expanding the range of EPNs and terpenoids used in agriculture. Research should also be conducted to investigate the potential for using entomopathogenic nematodes in combination with terpenoids in the same spray tank for field application, and to evaluate synergistic interactions to improve the targeting and effectiveness of both pest control agents.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13061143/s1, Figures S1 and S2: phylogenies; Figures S3–S6: chemical certifications, Table S1: differences of means data.

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