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# Essential Oil Volatiles as Sustainable Antagonists for the Root-Knot Nematode *Meloidogyne ethiopica*

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**Abstract:** Climate change is prompting a shift of tropical pests to locations with a previously lower probability of invasion. This is the case for root-knot nematodes, *Meloidogyne* sp., particularly of the tropical group. Among them, *M. ethiopica* is now considered a threat to European food security. The development of novel sustainable nematicides can be based on in vitro bioassays of highly active phytochemicals, e.g., volatiles from essential oils. However, a steady supply of nematodes is often very difficult and dependent on environmental conditions. In the present study, an in vitro co-culture system of *M. ethiopica* parasitizing hairy roots of *Solanum lycopersicum* was established, for the first time, to easily obtain populations of second-stage juveniles (J2). These were then used to screen the nematicidal activity of 10 volatile compounds characteristic of essential oils. Finally, information on the most successful compounds was reviewed to predict their environmental dispersion and ecotoxicological hazards. The *M. ethiopica* population obtained from the co-culture was morphologically similar to reported populations in natural conditions and could be accurately used in direct-contact bioassays. The aldehydes citral and citronellal induced complete mortality of the tested J2, at 1 mg/mL, while compounds from other chemical groups were not as successful. In comparison to commonly used commercial nematicides, citral and citronellal were less likely to accumulate in the water environmental compartment and have lower reported toxicities compared to aquatic organisms and to mammals. Overall, in vitro co-cultures showed the potential to expedite the screening and discovery of bioactive compounds as a contribution to the development of sustainable biopesticides, as well as to lower the impacts of modern farming on agroecosystems.

**Keywords:** aldehydes; biopesticides; citral; citronellal; essential oils; *Meloidogyne ethiopica*; predicted environmental distribution; root-knot nematodes; *Solanum lycopersicum*



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## 1. Introduction

Modern farming reached a turning point in which conventional practices, previously devised to strongly increase food production, must now transition to novel farming strategies that focus on enhancing the sustainability of agroecosystems, not damaging their ecological surroundings, and favoring productivity. Despite important efforts towards shifting from synthetic chemical pesticides to less damaging alternatives, there is still considerable concern for environmental persistence of pesticides and for ecological and human health

impacts [1]. Research now tackles the development of novel biopesticides based on plant and microbial secondary metabolites. Plant-derived crop protectants have a long history of use and research; however, there is, currently, only a limited number of these products being marketed, which is not nearly enough to meet the rising demand for biopesticides [2]. In this context, essential oils (EOs) are a group of ecologically safer alternatives that usually integrate successful biopesticide formulations. EOs are complex mixtures of volatiles that show a great potential to be used as biopesticides, given that they are easily obtained, mainly by hydrodistillation of plant material, and are generally composed of highly bioactive compounds, such as terpenes (mono-, sesqui-, and some diterpenes), phenols (mainly phenylpropanoids), and unrelated compounds that can also be present in high amounts [3]. Many biological activities are attributed to EOs, such as antifungal, antibacterial, acaricidal, insecticidal, antimalarial, antiviral, and nematicidal [4]. Against plant parasitic nematodes, the activities of EOs are generally attributed to their major compounds [5]. The volatile chemicals that generally compose EOs can be broadly found throughout the plant, bacterial, or fungal kingdoms. Some appear to have a suppressive activity against root parasitic nematodes on soil ecosystems, either directly affecting parasitic nematode populations or indirectly, by influencing rhizosphere microbiota [6–9]. Direct activity against root parasitic nematodes was mainly screened for the root-knot nematodes (RKN), *Meloidogyne* genus. These obligate sedentary plant endoparasites are distributed worldwide and can infect almost every species of vascular plants, being now recognized as the most important plant parasitic nematode for economic and research purposes [10]. The top four most dangerous species are *M. arenaria*, *M. incognita*, and *M. javanica* in tropical regions, and *M. hapla* in temperate zones; however, many other species are becoming increasingly threatening to modern farming, endangering the productivity of important crops. Among those species is *Meloidogyne ethiopica* (Whitehead, 1968), which was first described by Whitehead (in 1968) in the Mlalo region of Tanzania, and later redescribed (in 2004), being added to the European Plant Protection Organization (EPPO) alert list in 2011 [11–13]. *Meloidogyne ethiopica* was reported in Ethiopia, Kenya, Mozambique, Zimbabwe, Tanzania, South Africa, Chile, Brazil, Peru, Slovenia, Greece, and Turkey [11,12,14–18]. However, European populations were recently reclassified and identified as *Meloidogyne luci* through the application of biochemical and molecular analyses [19]. *Meloidogyne ethiopica* disrupts the development of the plant root system, leaving it deformed through the establishment of multiple small and large galls. Additionally, plants often exhibit aboveground symptoms, such as stunting and wilting. This species is considered harmful since it can parasitize numerous crops that include monocotyledons, dicotyledons, herbaceous, and woody plants [12,15,19–21]. This phytoparasite is widely distributed in vineyards in Chile and in kiwi fruit productions in Brazil, causing a reduction in crop growth and in fruit size and quality [15,22,23]. Due to its ability to adapt to temperate conditions and infect a wide variety of hosts, it poses a serious threat for agricultural production.

Research on plant parasitism of RKNs, particularly molecular and biochemical studies, lagged behind other fields of plant pathology because of their microscopic size, but also due to their complex infection cycle [10]. Considering that these nematodes are obligate phytoparasites, obtaining large amounts for experimentation is a considerably lengthy process, many times constrained by edaphic and environmental conditions, requiring many resources. Recently, a promising co-culture system was developed based on the use of hairy roots (HR) that allows for obtaining large amounts of genetically stable RKNs for experimentation [24]. In vitro-grown HRs can be suitable model host systems for research on RKN infection due to their rapid growth rate in plant growth regulator-free medium and a relative genetic and metabolomic stability. In these controlled environments, single variables can be manipulated, and nematode growth can be directly analyzed, which is difficult to perform under greenhouse or field conditions [25]. Ultimately, these co-cultures can provide more biomass by using fewer resources.

In the present work, in vitro co-cultures of *Solanum lycopersicum* L. (tomato) HRs with *M. ethiopica* were established to obtain large quantities of contamination-free J2 juveniles

for screening the nematicidal activity of ten bioactive volatiles as a contribution to the development of more sustainable biopesticides against this crop pest. Although the maintenance of in vitro hairy root/RKN co-cultures involves a number of resources and technical experience, its use allows for a reliable and steady supply of RKNs with a higher control of genetic variability and expedites screening bioactive volatiles for nematicidal activity.

## 2. Materials and Methods

### *2.1. Chemicals*

The volatiles used in the direct contact bioassays were analytical standards of the aldehydes citronellal (≥95%) and citral (mixture of *cis*- and *trans*-citral, ≥96%), the alcohols terpinene-4-ol (≥95%) and α-terpineol (≥96%), the ketones pulegone (98%) and 2-undecanone (99%), the hydrocarbons α-pinene (98%) and *p*-cymene (99%), and the phenolics *trans*-anethole (99%) and eugenol (≥98%), and were acquired from Sigma-Aldrich (St. Louis, MO, USA) (Figure 1). The HPLC-grade solvent methanol, used for stock solutions of the volatiles, was acquired from Fisher Chemicals (Fisher Chemicals, New Hampshire, USA).

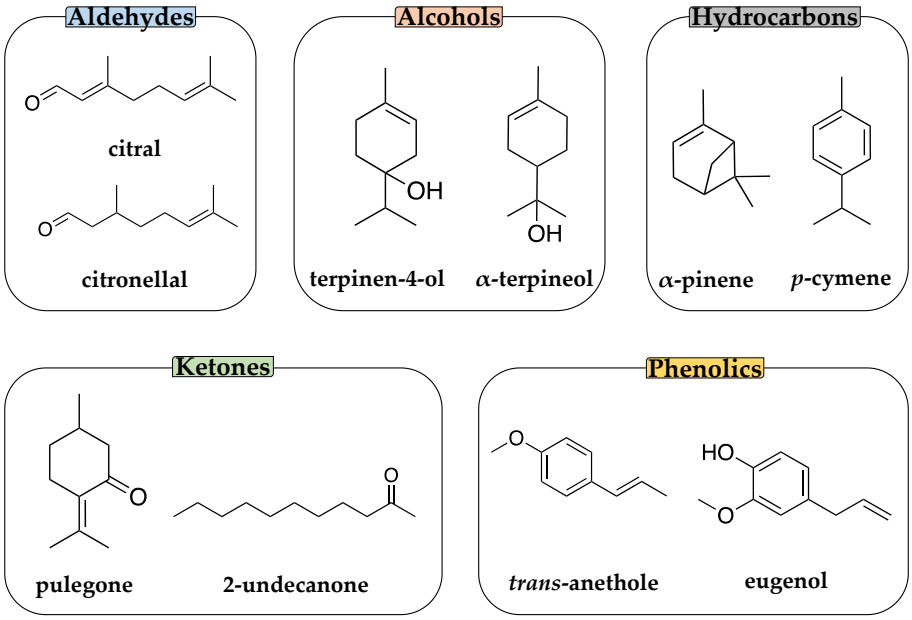



**Figure 1.** Chemical structures of the volatiles used in direct contact bioassays against second stage *Meloidogyne ethiopica* juveniles (J2).

### *2.2. Meloidogyne ethiopica Inoculum*

*Meloidogyne ethiopica* was kindly provided by the European Union Reference Laboratory (EURL) for Plant Parasitic Nematodes, ANSES-ILVO (Rennes, France), in the form of root galls, and is regularly maintained in tomato plants, *Solanum lycopersicum* L., cv. ox heart, as a reference isolate at the Plant Nematology Lab of the National Institute for Agrarian and Veterinary Research (INIAV, I.P.), in Oeiras, Portugal. For routine maintenance, 1 L black plastic pots are filled with a sterilized mixture of local soil, sand, and substrate (1:1:1), watered to 70% of maximum water holding capacity and maintained in these conditions for 1 week for soil stabilization. Tomato seedlings, obtained by germinating commercially acquired certified seeds on hydrated filter paper, are transferred to the pots and kept at 25 ± 1 °C. For nematode infection of the tomato seedlings, RKN egg masses were handpicked from the provided root galls and added to the soil in the vicinity to the roots of tomato plants at an early growth stage, namely at the second unfolded leaf on the main shoot stage of development, and maintained in a growth chamber at 25 ± 1 °C [26]. After ca. 60 days, tomato roots already display fully developed root galls with egg masses. These are handpicked from the tomato roots and the eggs are hatched in moist chambers at

25 ± 1 °C. The second-stage *M. ethiopica* J2 are counted by sampling five aliquots of 100 µL from the 5 mL solution where the eggs were hatched.

### *2.3. In Vitro Tomato Hairy Root Cultures*

In vitro hairy roots were established from *S. lycopersicum* cv. ox heart tomato fruits. Tomatoes in the first stage of maturity were obtained from local growers and washed with commercial detergent before being surface sterilized by immersion in ethanol 96% (Merck KGaA, Germany) for 10 min with frequent mixing. In aseptic conditions, tomatoes were rinsed 3 times in 100 mL of ultrapure sterile water. Following, ca. 0.5 cm-thick sections were obtained from the central portion of the fruit, and inoculated with *Rhizobium rhizogenes* ARqual strain [27]. Inoculation was performed by dipping a scalpel in a suspension of *R. rhizogenes* and then using it to wound the aseptic tomato sections. The bacterial suspension was obtained by growing *R. rhizogenes* overnight and diluting it, when $A_{600}$ = 0.6, at 1:10 (*v*/*v*) in liquid Schenk and Hildebrandt (SH) medium with 30 g/L sucrose, pH = 5.6 [28]. Inoculated segments were then placed on solid SH medium (8 g/L agar) and co-cultivated with the bacteria for 4–5 days, being then transferred to solid SH medium supplemented with 150 µg/mL each of cefotaxime and carbenicillin (Sigma-Aldrich, St. Louis, MO, USA), to eliminate the bacteria. Tomato sections were weekly sub-cultured to fresh culture medium (with antibiotics) for over 3 months. Any HRs that emerged from the inoculated tomato sections (or from germinated immature seeds) were excised and cultured in antibiotic-free solid SH medium. After three sub-culture periods, HR pieces were transferred to liquid SH medium and maintained on an orbital shaker at 80 r.p.m. and 25 ± 1 °C. Monthly sub-culturing was performed by aseptically removing a portion of the root clump and transferring to fresh culture medium. Tomato HR cultures were maintained in darkness at 25 ± 1 °C throughout.

### *2.4. Co-Cultures of Solanum lycopersicum Hairy Roots with Meloidogyne ethiopica*

Co-cultures were established by infecting tomato HRs with aseptic suspensions of *M. ethiopica* J2. The previously obtained J2 suspensions were sieved on a sterilized 20 µm mesh steel sieve, diameter 11.5 cm, and height 6 cm (Retsch GmbH, Haan, Germany), and sterilized with 5 mL of a 10% hydrogen peroxide (30%, Merck KGaA, Germany) solution for 5 min in a flow hood. Afterwards, the excess sterilizing agent was removed with three washes of 15 mL of sterilized ultrapure water and the RKNs were retrieved by pipetting with 500 µL of sterilized ultrapure water. The contamination-free suspensions of *M. ethiopica* juveniles were distributed on petri dishes (ca. 100–150 J2 per dish) containing freshly inoculated tomato HRs on solid SH medium with 30 g/L sucrose, pH = 5.6 (8 g/L agar) and left in darkness at 25 ± 1 °C for over 2 months to ensure population development and reproduction. Every 4 weeks, in vitro co-cultures were sub-cultured by aseptically transferring a portion of the parasitized root clump to fresh solid SH medium and maintained as described above. Co-cultures were transferred to liquid SH medium after ca. 6 months in solid medium and maintained in darkness at 25 ± 1 °C and 80 r.p.m., with monthly sub-culturing by refreshment of the aseptic culture medium.

To obtain suspensions of *M. ethiopica* J2 from these co-cultures, galled HRs were excised and the nematode eggs extracted by vigorous agitation for 5 min in a 0.52% (*v*/*v*) sodium hypochlorite (NaOCl) solution [29]. This mixture was then sieved through a 75 µm mesh steel sieve to retain root debris, and the eggs were recovered in a 20 µm mesh steel sieve. These were rinsed thoroughly with ultrapure water and hatched in moisture chambers as described above.

### *2.5. Characterization of M. ethiopica Life Stages in the Co-Culture*

To isolate and identify the life stages of *M. ethiopica*, root clumps were picked from the aseptic co-cultures established in liquid culture medium. Nematodes in suspension in the co-culture medium were counted 4 weeks after refreshment of the medium, and nematodes in the root clumps were extracted with a 0.52% (*v*/*v*) NaOCl solution and

counted, as described above, in a minimum of 6 replicates. The quantification of nematodes and/or assessment of survival rates was performed with an Olympus SZX12 (Tokyo, Japan) stereomicroscope. For nematode morphological traits, at least 10 specimens of each life stage were heat-killed and placed in a drop of water on a glass slide and observed using an Olympus BX-51 bright field light microscope (Hamburg, Germany) and photographed with an Olympus DP10 digital camera. When needed, co-culture root clumps were treated with acid fuchsin to stain nematodes [30]. For this purpose, root pieces were washed for 4 min in a 1.5% NaOCl solution, rinsed in distilled water, and added to boiling staining solution for 30 s. The staining solution was composed of 1 mL of stain [3.5 g of acid fuchsin (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 250 mL of acetic acid (Fisher Chemicals, Newington, NH, USA) and 750 mL of distilled water] in 30 mL of distilled water. After cooling to room temperature, the excess stain was removed under running tap water and the roots were retained in a 20 µm mesh steel sieve. The roots were placed in glycerin (60%), acidified with 5 M HCl (0.1%), and the nematode life stages were picked into microscope slides for measuring.

### *2.6. Direct Contact Bioassays with Volatiles*

Stock solutions of the chemical standards were prepared in methanol at a 20 mg compound/mL of methanol and kept at −20 °C until needed for the bioassays. Aliquots of 95 µL of a suspension of *M. ethiopica* J2, at 500 ± 50 nematodes/mL, were distributed per well of 96-well microtiter plates (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). To obtain a final concentration of 1 mg/mL, 5 µL of each stock solution were added to the wells. Ten replicates were performed for each compound in two separate bioassays. Control and blank wells were also performed by adding 5 µL of methanol or ultrapure water, respectively, to assess mortality induced by methanol and the natural mortality. The microtiter plates were then mixed in an orbital shaker (IKA labortechnik, Staufen, Germany) at 800 cycles/min for 1 min, covered with plastic film and aluminum foil, and maintained at 25 ± 1 °C in an orbital shaker at 50 r.p.m. for 96 h. Live and dead nematodes were counted every 24 h, as described above, and J2 immobility was tested by mechanically probing. Mortality was considered if juveniles showed no movement after mechanical stimulation.

### *2.7. Predicted Environmental Fate of the Nematicidal Volatiles*

The potential environmental fate of the volatiles with the highest nematicidal activity was determined through the predictive equilibrium criterion model suggested by Mackay et al. [31], and compared to that of the synthetic nematicides oxamyl, metham sodium, and fluopyram. Supported by this model, the predicted environmental distribution (PED) percentages were obtained for citral and citronellal in the environmental compartments air, water, soil, and sediments by using the freely available Level I Mackay Fugacity Model beta version 4.31, Trent University, Canada [32]. The model for this level predicts a situation in which a fixed quantity of compound, namely an illustrative 100,000 kg, is introduced to a closed system under steady-state and equilibrium conditions at 25 °C. The chemical parameters needed from each compound, namely, molecular mass (g/mol), melting point (°C), vapor pressure (Pa), solubility in water (mg/L), air–water partition coefficient or Henry's law constant (Pa.m$^3$/mol), n-octanol/water partition coefficient (log value of Kow), and soil organic carbon/water partition coefficient (Koc) were retrieved from PubChem online database [33] and PPDB: the Pesticide Properties Database [34] (Table 1).

**Table 1.** Physico-chemical properties of the volatiles and nematicides used to perform the Level I Mackay Fugacity Model [32], namely, molecular mass (g/mol), melting point (°C), vapor pressure (Pa), solubility in water (mg/L), air–water partition coefficient or Henry's law constant ($Pa.m^3/mol$), n-octanol/water partition coefficient (logKow), and soil organic carbon/water partition coefficient (Koc). Data were retrieved from the PubChem database [33] and PPDB: the Pesticide Properties Database [34].

| | **Volatiles** | | **Nematicides** | | |
|---|---|---|---|---|---|
| | **Citronellal** | **Citral** | **Oxamyl** | **Metham Sodium** | **Fluopyram** |
| CAS number | 106-23-0 | 5392-40-5 | 23135-22-0 | 137-42-8 | 658066-35-4 |
| Molecular mass (g/mol) | 154.25 | 152.23 | 219.26 | 129.19 | 396.76 |
| Melting point (°C) | −16.0 | −10.0 | 98.5 | 88.5 | 117.5 |
| Vapor pressure (Pa) | 33.331 | 12.172 | $1.800 \times 10^{-5}$ | 0.058 | $1.200 \times 10^{-6}$ |
| Solubility in $H_2O$ (mg/L) | 70.2 | 1340.0 | 148,100.0 | 578,290.0 | 16.0 |
| Henry's law constant ($Pa.m^3/mol$) | 73.235 | 1.383 | $2.670 \times 10^{-7}$ | $1.280 \times 10^{-5}$ | $2.980 \times 10^{-5}$ |
| $logK_{OW}$ (unitless) | 3.53 | 2.76 | −0.44 | −2.91 | 3.30 |
| $K_{OC}$ (unitless) | 650 | 83 | 15 | 18 | 279 |

### *2.8. Acute Toxicity Thresholds of the Nematicidal Volatiles*

Data on the toxicological and ecotoxicological parameters of the nematicidal volatiles and the synthetic nematicides oxamyl, metham sodium, and fluopyram on aquatic organisms and on mammals were retrieved from PubChem [33], ECHA, the European Chemicals Agency [35], and PPDB: the Pesticide Properties Database [34].

### *2.9. Data Treatment and Statistical Analysis*

The percentage mortality in each well was determined with the formula: mortality % = [(dead J2 RKNs)/(live + dead J2 RKNs)] × 100. To determine the mortality caused by each compound, mortality percentages were corrected with the Schneider-Orelli formula: corrected mortality % = [(mortality % in treatment − mortality % in control)/(100 − mortality % in control)] × 100 [36]. The nematicidal strength of the tested compounds was classified, at each time point, as complete (100%), strong (80–99%), moderate (61–79%), weak (40–60%), and low or inactive (>40%) [37]. Statistical analysis was performed with SPSS version 29 statistics software. Statistical significance was determined with one-way ANOVA, and individual means were compared using the Tukey's post hoc test with $p < 0.05$, the Shapiro–Wilk test ensured data normality, and the Browns–Forsythe test was used for homoscedasticity. Results are presented as the average and standard error of 10 replicates.

## 3. Results

The establishment of HRs was successful for *S. lycopersicum* cv. ox heart. Between three and four weeks after transformation with the bacteria, small roots were seen emerging from the infected tomato sections, mainly in seedlings that germinated from sectioned immature seeds of the tomato portions (Figure 2a). Tomato HRs were transferred to fresh SH medium, and within four weeks, the petri dishes (nine cm of diameter) were fully colonized (Figure 2b). For the development of co-culture systems, aseptic *M. ethiopica* J2 were added to freshly sub-cultured tomato HRs. Within one to two months, multiple root galls developed and egg masses could be distinguished as dark brown spots along the lighter HRs. This phenotype was observed in co-cultures grown in both solid and liquid culture medium (Figure 2c,d). Under a stereomicroscope, egg masses could be seen being produced by the *M. ethiopica* females encased in the root galls (Figure 2d–f).

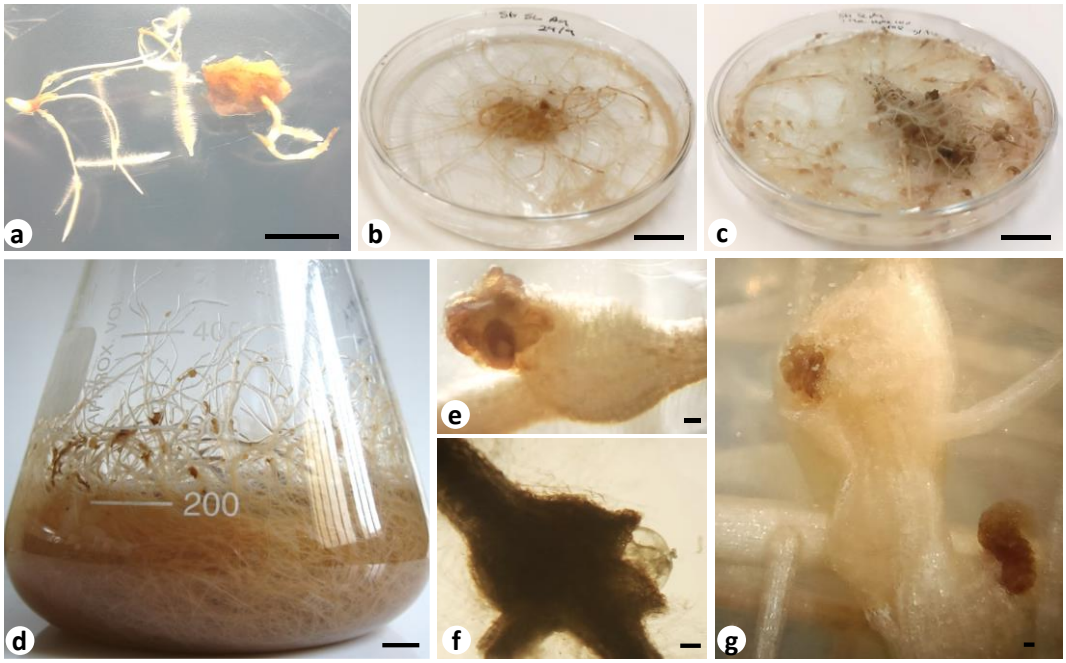



**Figure 2.** Hairy roots (HR) of *Solanum lycopersicum* obtained from aseptic sections of tomato fruits infected with *Rhizobium rhizogenes* (**a**) and grown on solid Schenk and Hildebrandt (SH) medium [28] (**b**); co-cultures of *S. lycopersicum* HR with *Meloidogyne ethiopica* in solid (**c**) and liquid (**d**) SH medium and detailed micrographs of in vitro root galls displaying the characteristic egg mass (**e**), female body (**f**), and singular eggs released into the culture medium (**g**). Scale bars 1 cm (**a**–**d**), 100 µm (**e**–**g**).

The RKN population that developed in the co-cultures of tomato HRs with *M. ethiopica* was characterized for the cultures maintained in liquid culture medium. Approximately 35 ± 5 nematodes per mL of culture medium, namely juveniles and some adult males, were counted in suspension in the liquid culture medium.

However, the nematodes extracted from the co-culture root clumps were quantified at 1621.6 ± 156.0 eggs/g co-culture fresh weight (FW) and 360.4 ± 56.9 RKNs/g co-culture FW. To assess possible deviations in RKN morphology, due to their development in an in vitro environment, the different nematode life stages of the RKNs extracted from these co-cultures were morphologically and morphometrically characterized and compared to previously reported morphological parameters from RKNs collected in field infections under natural conditions. The *M. ethiopica* J2 analyzed varied between 372.1 and 461.6 µm in length and were vermiform, slender, and clearly annulated (Table 2, Figure 3a,b). Their head region was not set off from the body and was lacking annulation. The stylet length varied between 14.0 and 15.5 µm with a clearly cylindrical shaft, and the cone had an evenly increasing width. The stylet knobs were rounded and set off from the shaft. The excretory pore was distinct and the hemizonid was located anteriorly to the excretory pore. The tail length varied between 43.9 and 64.1 µm and the tail was delicate, with a rounded tip and distinctive hyaline terminus with ca. 10.8 ± 0.6 µm (Table 2, Figure 3a,b). When compared to populations from natural conditions (*in vivo*), the J2 showed no substantial morphological or morphometric differences in the parameters measured (Table 2). *Meloidogyne ethiopica* males isolated from the co-cultures were slightly longer than reported in the literature, showing 1849.3 ± 130.8 µm in comparison with an expected 1171.0 ± 3.0 µm (Table 2, Figure 3c–e) [12]. However, similarly to what was previously reported, males had a vermiform body and head slightly set off with a rounded head cap. The stylet varied from 22.7 to 26.9 µm in length, and was robust and large, with a straight and pointed cone and a shaft with a gradually increasing width. The stylet knobs were pear-shaped and posteriorly sloping. The tail was relatively short (11.7 ± 0.3 µm) and the spicules were thick and curved (Figure 3c–e). With the exception of its superior length and the parameters

calculated using this measurement, males showed comparable characteristics to natural populations (Table 2). The females isolated from co-culture root galls were white and pear-shaped with a prominent neck. The perineal pattern was oval to squarish, the striae was coarse and widely separated, and the phasmids were distinct.

**Table 2.** Morphometric parameters determined for second stage juveniles (J2) and adult males of *Meloidogyne ethiopica* obtained from the co-cultures of tomato hairy roots with *M. ethiopica* (in vitro) or retrieved from bibliographic sources (in vivo) [12].

| | J2 | | Males | |
|---|---|---|---|---|
| | **In Vitro** | **In Vivo** | **In Vitro** | **In Vivo** |
| Body length (µm) | 412.6 ± 10.5 (372.1–461.6) | 468.0 ± 3.0 (326.0–510.0) | 1849.3 ± 130.8 (1172.4–2313.9) | 1171.0 ± 3.0 (890.0–1500.0) |
| a [1] | 24.4 ± 0.3 (22.8–26.4) | 24.0 ± 0.3 (21.3–28.2) | 42.4 ± 1.2 (39.2–52.7) | 27.7 ± 0.8 (24.8–31.0) |
| c [2] | 7.7 ± 0.4 (5.8–9.3) | 4.8 ± 0.1 (3.9–6.4) | 155.9 ± 10.3 (99.9–184.7) | 114.0 ± 12.2 (69.5–147.2) |
| Greatest body diam. (µm) | 16.9 ± 0.5 (14.1–19.0) | 20.0 ± 0.3 (15.0–22.0) | 42.9 ± 2.0 (30.2–52.7) | 48.0 ± 0.8 (32.0–59.0) |
| Tail length (µm) | 54.1 ± 2.1 (43.9–64.1) | 62.0 ± 0.6 (52.0–72.0) | 11.7 ± 0.3 (9.4–12.8) | 13.4 ± 0.5 (10.2–17.0) |
| Stylet length (µm) | 14.9 ± 0.2 (14.0–15.5) | 12.2 ± 0.1 (11.0–14.0) | 25.3 ± 0.4 (22.7–26.9) | 24.8 ± 0.6 (23.0–27.0) |
| DGO [3] (µm) | 2.8 ± 0.1 (2.1–3.3) | 2.6 ± 0.1 (2.0–3.0) | 4.3 ± 0.1 (3.8–4.9) | 2.5 ± 0.1 (2.0–3.5) |
| Hyaline tail terminus (µm) | 10.8 ± 0.6 (7.3–12.6) | 13.5 ± 0.2 (12.0–15.0) | - | - |

[1] Ratio of body length over greatest body diameter, [2] ratio of body length over tail length, and [3] dorsal gland orifice.

The dorsal arch was moderately high, rounded to squarish, and the lateral lines absent (Figure 3f,g). As above, this morphology was consistent with the descriptions reported for this species [12]. Thus, the nematodes produced by the established co-cultures were considered to be representative of natural populations.

For the direct-contact bioassays, mortality in blank wells (methanol toxicity) was similar to natural mortality, throughout the time-course study, which indicates that, at the percentage used, methanol induced no substantial mortality in *M. ethiopica*. The detected natural mortality was 2.1 ± 0.1, 3.5 ± 0.6, 3.5 ± 0.7, and 5.5 ± 0.2%, while methanol induced 2.8 ± 0.1, 3.0 ± 0.3, 3.2 ± 0.1, and 5.3 ± 0.9% mortality on J2, at 24, 48, 72, and 96 h, respectively. The volatiles assayed against *M. ethiopica* J2 showed very different activities. The monoterpene aldehydes tested, citronellal and citral, showed similar activities, namely very strong to complete activity (99.0 ± 0.2 and 100 ± 0%, respectively) after 24 h of direct contact, and maintained complete activity until the end of the time-course study (Figure 4a,b). The monoterpene alcohol terpinene-4-ol showed a peak of strong activity at 48 h (60.6 ± 1.0%), but decreased to low activities after 72 h (2.5 ± 0.2%) and 96 h (1.8 ± 0.2%) (Figure 4c). Conversely, α-terpineol imposed a strong toxicity after 24 h of direct contact (65.5 ± 0.4%), but decreased considerably until the end of the time-course study (Figure 4d). The monoterpene ketone pulegone was shown to be inactive, while 2-undecanone showed a peak of strong activity at 48 h (89.7 ± 0.8%) and 72 h (87.2 ± 0.1), but decreased to ca. 34% after 96 h of direct contact (Figure 4e,f). The hydrocarbons α-pinene and *p*-cymene showed mostly weak to moderate activities (Figure 4g,h). The phenolic volatiles had different toxicities towards *M. ethiopica* juveniles, while *trans*-anethole showed only a moderate activity (the highest activity being 32.6 ± 4.5%, at 48 h of direct contact), eugenol induced very high activities, and after 48 h of direct contact, induced complete immobility of the nematode body (Figure 4i,j). However, this nematotoxic activity was reduced to 79.6 ± 1.3% after 96 h.

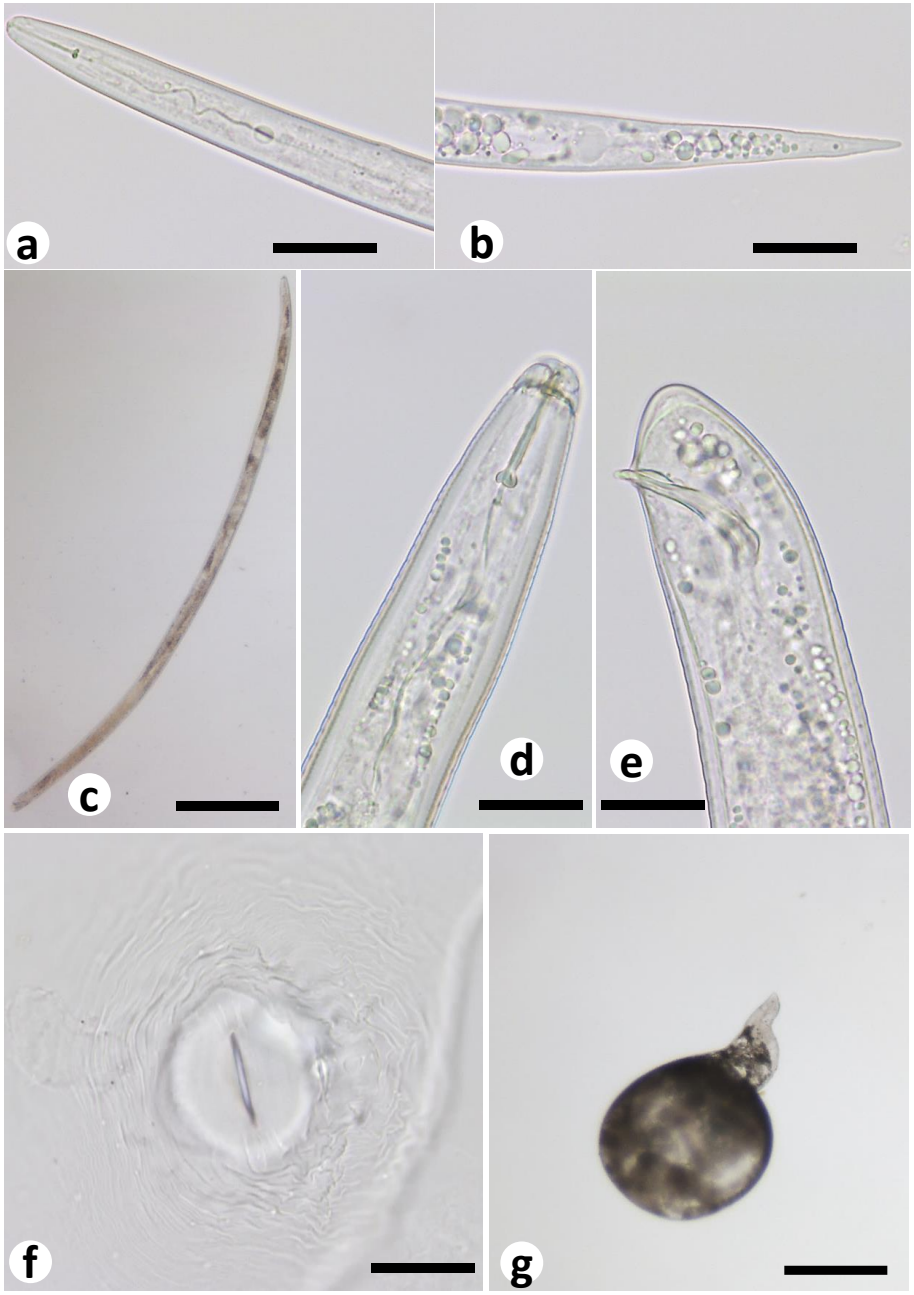



**Figure 3.** Micrographs of root-knot nematode life stages obtained from the co-cultures of *Solanum lycopersicum* hairy roots with *Meloidogyne ethiopica*. Second stage juvenile (J2) anterior region (stylet, excretory pore and hemizonid) (**a**) and tail region (rectum and hyaline part) (**b**), adult male (**c**) anterior (**d**) and posterior region (spicules) (**e**); and perineal pattern (**f**) of the female (**g**) commonly used for species identification. Scale bars 20 µm (**a**,**b**,**d**–**f**), 200 µm (**c**,**g**).

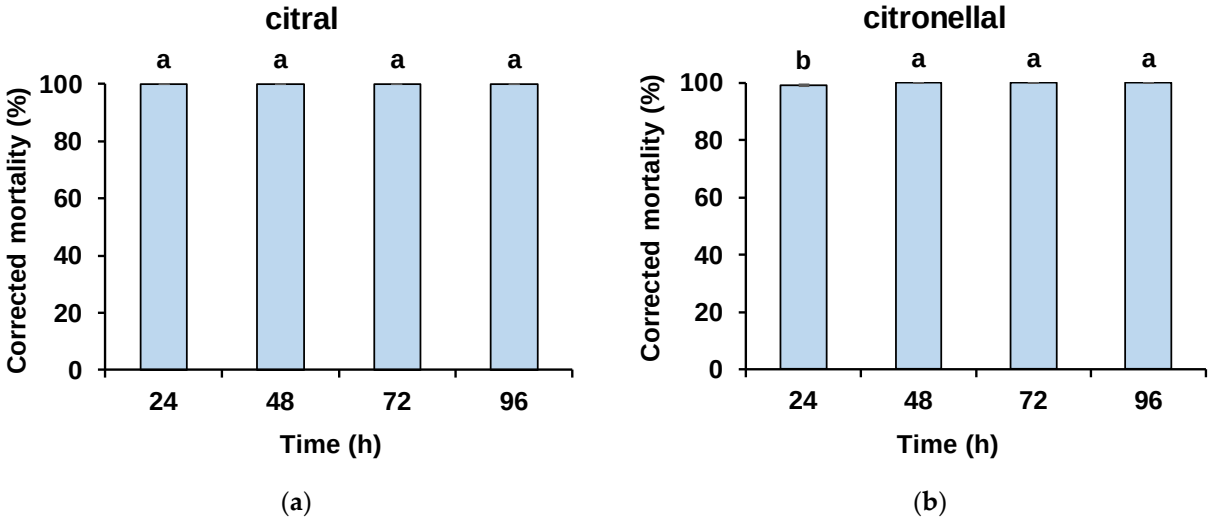
A predictive approach was used as an indicator of the favored affinities of the most active volatiles to the main environmental compartments. PED percentages were determined for each compound and compared to those of three commercial nematicides, namely, oxamyl, metham sodium, and fluopyram, in order to assess if they could be a more sustainable alternative (Table 3). Citronellal showed a higher predicted affinity to air (86%) than to the soil (8%) or water (6%) environmental compartments, which suggests that it is easily volatilized. Alternatively, citral showed a higher predicted affinity to the water (68%) than to the air (19%) or soil (12%) environmental compartments. In comparison, oxamyl,

metham sodium, and fluopyram displayed a higher predicted affinity to water (97, 96, and 62%, respectively) and soil environmental compartments (3, 4, and 37%, respectively).

**Table 3.** Predicted environmental distribution (PED) percentages in the air, water, soil, and sediments environmental compartments computed through the Mackay fugacity model [31], the reported acute toxicities for aquatic organisms (fish, daphnia, and algae) (median lethal dose, $LD_{50}$, mg/L), and oral and dermal acute toxicities for mammals (median lethal dose, $LD_{50}$, mg/kg) obtained from PubChem online database [33], for the volatiles citronellal and citral and the nematicides oxamyl, metham sodium, and fluopyram.

| | Volatiles | | Nematicides | | |
|---|---|---|---|---|---|
| | **Citronellal** | **Citral** [1] | **Oxamyl** | **Metham Sodium** | **Fluopyram** |
| **PED (%)** | | | | | |
| Air | 85.84 | 19.07 | $5.20 \times 10^{-6}$ | $2.49 \times 10^{-4}$ | $3.71 \times 10^{-4}$ |
| Sediments | 0.18 | 0.27 | 0.07 | 0.08 | 0.83 |
| Soil | 8.16 | 12.26 | 3.14 | 3.74 | 37.28 |
| Water | 5.81 | 68.38 | 96.79 | 96.17 | 61.86 |
| **Ecotoxicology (mg/L)** | | | | | |
| Fish | 22.00 [2] | 6.10 [3] | 3.13 [3] | >0.18 [4] | >0.98 [5] |
| Daphnia [6] | 8.68 | 10.00 | 0.32 | 0.99 | >100.00 |
| Algae | 13.33 [7] | 5.00 [7] | 0.93 [8] | 1.08 [8] | >1.13 [9] |
| **Toxicology (mg/kg)** | | | | | |
| Oral [10] | >5000 | 6800 | 2.5 | 896 | >2000 |
| Dermal [10] | >2500 | >1000 | 5000 | 2000 | 2000 |

[1] Citral occurs as a mixture of the stereoisomers geranial (*trans*-citral) and neral (*cis*-citral), more commonly found in natural conditions; ecotoxicological $LD_{50}$ values were reported for the following model organisms [2] *Oryzias latipes*, [3] *Oncorhynchus mykiss*, [4] *Lepomis macrochirus*, [5] *Coleonyx variegatus*, [6] *Daphnia magna*, [7] *Selenastrum capricornutum*, [8] *Pseudokirchneriella subcapitata*, and [9] *Skeletonema costatum*; and [10] toxicological $LD_{50}$ values of volatiles and nematicides were reported for rat, with the exception of the acute dermal $LD_{50}$ value for citronellal, which was reported in rabbit.




(**a**) (**b**)

**Figure 4.** *Cont.*

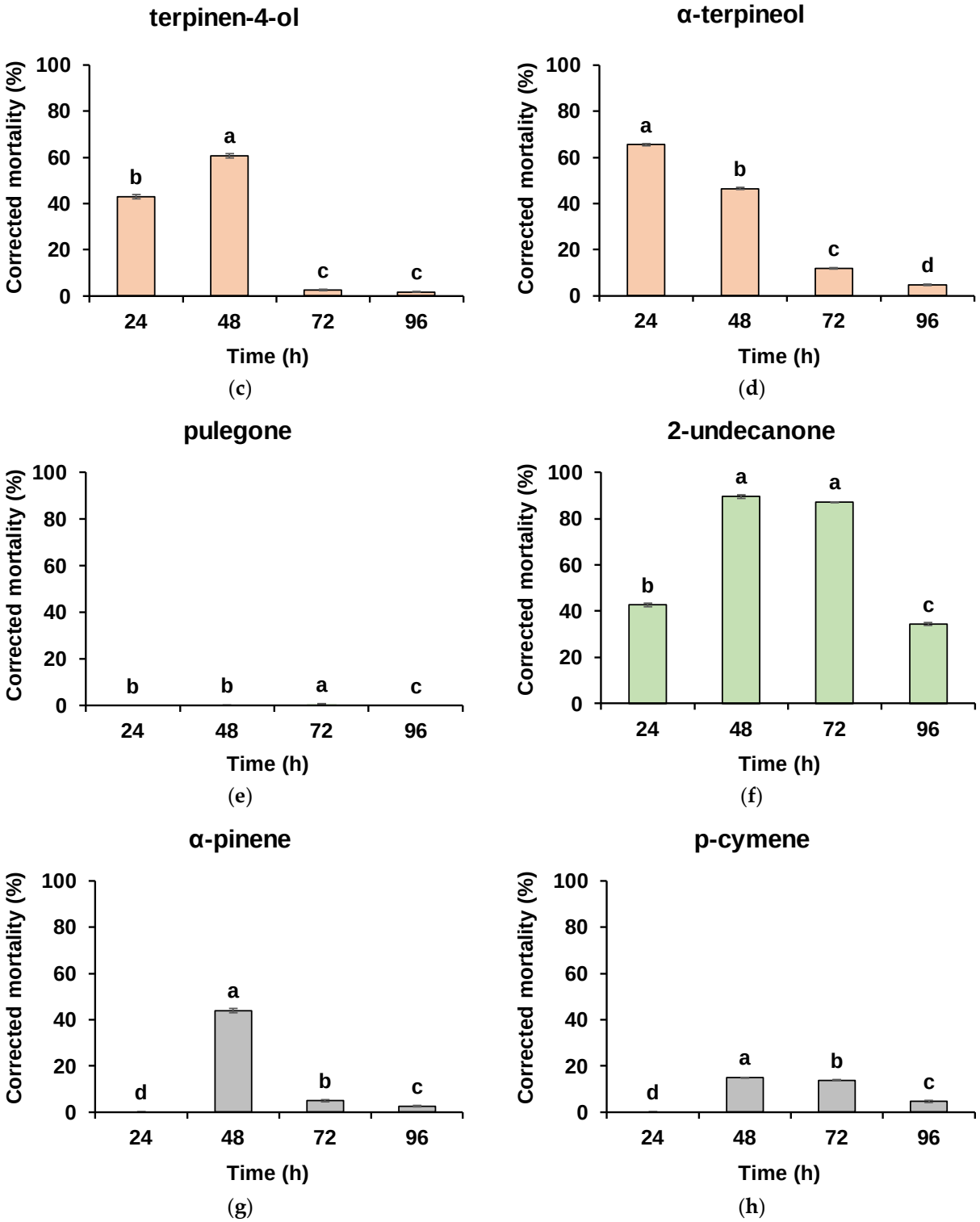



**Figure 4.** *Cont.*

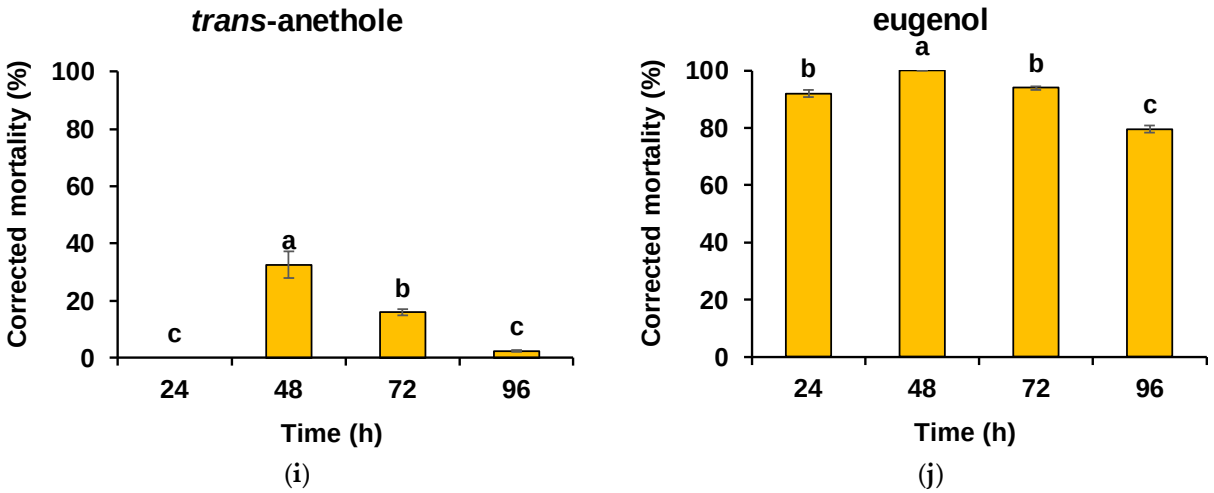



**Figure 4.** Corrected mortality (%) values obtained for the aldehydes citral (**a**) and citronellal (**b**), alcohols terpinene-4-ol (**c**) and α-terpineol (**d**), ketones pulegone (**e**) and 2-undecanone (**f**), hydrocarbons α-pinene (**g**) and *p*-cymene (**h**), and the phenolics *trans*-anethole (**i**) and eugenol (**j**), in direct contact bioassays with *Meloidogyne ethiopica* for 24, 48, 72, and 96 h. Data are presented as means ± standard error of the independent biological replicates. Different letters indicate statistically significant differences ($p < 0.05$).

Due to the PED percentages of these compounds, acute toxicity values for model organisms of water environments were compiled from online databases and compared. Citral and citronellal are mostly reported to show higher lethal dose values than synthetic nematicides. Commercial nematicides were reported to have the highest toxicity to fish, daphnia, and algae (for detailed model species reported see Table 3 footnote), with the exception of fluopyram toxicity against daphnia (>100 mg/L), which indicates its probable safety for crustacean organisms.

In what concerns their safety to human health, the reported acute oral toxicities for mammals are generally lower for citral and citronellal, with the exception of fluopyram, which is reported to possess comparable oral and dermal toxicities, suggesting a similar hazard potential.

## 4. Discussion

The RKN *M. ethiopica* is a highly damaging plant pest in the tropical regions of Africa and South America and may become a devastating pest to the EU, given that its closest relative *M. luci* was already identified as thriving in European countries (Italy, Greece, Slovenia, Portugal and Turkey). Uncovering sustainable management strategies in a timely manner is thus dependent on a steady and reliable supply of its different life stages for testing. The co-cultures established in the present work offer a high-yielding biotechnological laboratory tool with the potential to produce a constant amount of *M. ethiopica* eggs, roughly at a four to five-week interval.

The use of in vitro roots transformed with *R. rhizogenes* is not new and was previously studied in combination with RKNs to uncover important mechanisms of plant–nematode interactions, e.g., in analyzing the biochemical mechanisms of RKN parasitism [38], for characterizing and/or validating candidate genes for host resistance to RKNs [39–42], in propagating RKN resistant plant genotypes [43], in understanding the processes of RKN infection after knocking out specific stress-related or putative susceptibility genes [44], or for the general maintenance of RKN collections [45–47]. However, resorting to plant–nematode co-cultures as a tool to uncover bioactive compounds or mixtures for the design of safer biopesticides is less reported in the literature. In a previous work, in vitro cultures of potato (*Solanum tuberosum*) HRs were established and infected with *M. chitwoodi*, a highly damaging RKN already spread through America, Europe, and Africa, in a true

co-culture system, to provide large amounts of RKN eggs for the screening of nematicidal EOs [24]. In a follow-up study, 56 EOs from 16 plant families were bioassayed against *M. chitwoodi*, to understand how nematicidal EOs inhibit RKN egg hatching under in vitro conditions [29]. From the bioassayed EOs, those of *Dysphania ambrosioides*, *Filipendula ulmaria*, *Ruta graveolens*, *Satureja montana,* and *Thymbra capitata* showed the highest anti-hatching activities [29]. The main compounds of these EOs, namely, ascaridol, carvacrol, *p*-cymene, isoascaridol, methyl salicylate, γ-terpinene, and 2-undecanone, were believed to be responsible for RKN hatching inhibition. However, instead of resorting to commercial standards of these volatiles, the authors performed column chromatography to isolate the hydrocarbon (apolar volatiles) from the oxygen-containing compound (polar volatiles) fractions of the EOs to better determine the origin of its respective anti-hatching properties in the complex mixture of the EO. For the EOs of *Cymbopogon citratus*, *Origanum vulgare*, *Satureja montana, Thymbra capitata,* and *Thymus caespititius*, the anti-hatching activity was seen to mainly reside in the oxygen-containing compounds fraction. Notably, citral was included in these types of compounds as a component of *C. citratus* EO. Conversely, the hydrocarbon fractions of these EOs induced only a moderate activity, not reaching complete hatching inhibition at the highest concentration, at 2 µL/mL. In almost all the analyzed hydrocarbon fractions, *p*-cymene featured as a major compound. Although in the present work, anti-nematode activity was determined from J2 mortality bioassays and not from egg hatching inhibition assays, citral and *p*-cymene showed activity patterns similar to the ones obtained in the previous work.

Despite the easy availability of EOs, much of the research performed focuses on screening commercial standards of the EO main compounds, mainly due to the complex nature of an EO, which is generally composed of more than 20 different compounds in variable proportions. The aldehydes citral and citronellal differ structurally in the presence of a double bond. For these volatiles, much of the literature found was linked to the EO of species of the genus *Cymbopogon*, where they can exist in high amounts. The compounds emitted by *C. nardus* were seen to be highly active against *M. incognita,* and citronellal was identified as one of its major volatiles [48]. In another study, citronellal, as a major compound of the *Corymbia citriodora* EO (nematicidal against *M. incognita*), was assayed in silico for its affinity in binding to seven target proteins through a molecular docking approach [49]. A very good binding energy value was computed for the complex of citronellal with the odorant response gene 1 (ODR1), a receptor that is known to regulate nematode chemosensory functions. Although this type of study is fundamentally predictive, it provides a good contribution for understanding the bioactivity of aromatic oils and their compounds.

For citral, strong activities were previously mainly reported against *M. incognita* [50–52] and *M. graminicola* [53]. Both under in vitro and in vivo conditions, the activities obtained were sometimes comparable to commercial nematicides, such as carbofuran. In another study, the authors tackled the hypothesis of enhancing the already high nematicidal activity of citral against *M. incognita* by the activity-guided alteration of its chemical structure [54]. Of the alterations performed, the synthetized citral epoxide induced an increased J2 mortality and egg hatching inhibition. In comparison to citral, this compound effectively doubles its number of oxygen atoms, which further supports the notion that nematicidal activity may reside on specific oxygen-rich chemical structures. In another effort to enhance the nematicidal strength of citral, its synergistic interaction with undecan-2-one (or 2-undecanone), a major compound of *Ruta* spp. EOs, was analyzed against *M. incognita* [36,55,56]. The authors found that a 2:1 mixture of citral with 2-undecanone acted synergistically in reducing the number of galls and eggs of *M. incognita* in tomato roots. The toxicity mechanism induced by this interaction was not detailed; however, 2-undecanone is known to induce strong activities against the RKN. For example, against *M. incognita*, *M. javanica,* or *M. arenaria*, 2-undecanone extracted from plant (*R. chalepensis* or *R. graveolens*) or bacterial sources was seen to cause toxicity and influence nematode mobility and behavior [25,29,56–59]. Furthermore, when applied in sublethal doses, this aliphatic ketone appears to strongly attract *M. incognita*, which can contribute to the development of nematicidal formulations through a mechanism of disruption of nematode

chemotaxis in the root system [60]. Despite its strong activity, the nematicidal mode of action of 2-undecanone is known to be different from that of commercial nematicides, acting by directly degenerating the pseudocoel cells instead of influencing acetylcholinesterase activity [61].

The phenylpropanoid eugenol also showed high activities against *Meloidogyne* spp. by inhibiting egg hatching and differentiation in *M. javanica* [62], by acting directly on the survival of *M. incognita* or *M. graminicola*, when applied as a component of *Agastache rugosa* or *Syzygium aromaticum* EOs, respectively [53,63], by reducing *M. arenaria* gall formation in tomato [64], or even by enhancing the activity of other EO compounds in a 1:1 synergistic interaction [65].

From an ecological point of view, the high affinity of the nematicidal aldehydes citronellal and citral to the air environmental compartment sets them apart from the synthetic nematicides oxamyl, metham sodium, or fluopyram, whose affinity is mainly to the water environmental compartment. This is mainly due to their volatile nature, which may indicate a lesser persistence in the interstitial water in the soil or in bodies of water than the commercial nematicides. The exception may be citral, which also shows affinity to the water environmental compartment; however, its toxicity to aquatic organisms is lower than that of most commercial nematicides. Towards mammals, these aldehydes are reported to show much lower oral toxicities than the nematicides oxamyl, metham sodium, or fluopyram, and comparable dermal toxicities.

Overall, the present work differs from previous studies and related works by presenting a methodology to easily and reliably screen the activity of biocidal volatiles on developmental stages of *M. ethiopica* and to indicate the possible environmental fates and toxicity potential of the most successful volatiles in comparison to currently used nematicides.

## 5. Conclusions

Co-cultures of hairy roots with RKNs can be powerful biotechnological tools to assist in screening ecologically safer alternatives to commercial pesticides, not only for providing a constant and endless supply of several life stages of the phytoparasite, but also because they occupy less space and can be easily manipulated. Using this approach, a representative population of *M. ethiopica* was easily obtained from co-cultures with tomato HRs and used to identify the highly active aldehydes citronellal and citral as toxic against the RKN J2s. These volatiles, characteristic of EOs, stood out for their high activities against *M. ethiopica* and their lower predicted environmental impacts, encouraging their selection for the development of sustainable nematicidal formulations. The methodology pipeline laid out by this work has the potential to be applied to other phytoparasitic nematodes and can greatly expedite the screening of active compounds against nematode plant pests.

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