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ORIGINAL PAPER

Mode of action and ecotoxicity of hexanoic and acetic acids on *Meloidogyne javanica*

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Abstract

The main goal of the present study was to evaluate the hexanoic acid (HEX) and acetic acid (ACET), two active ingredients of the nematicidal *Melia azedarach* fruits water extract (MWE), for use on root knot nematodes control. We studied the effect of the acids on various growth stages of the phytoparasitic nematode *Meloidogyne javanica*, along with the phytotoxicity on tomato plants, their fate in soil and ecotoxicology, including non-target soil nematode and microbial communities. The $EC_{50/4d}$ values established for paralysis activity on second-stage juveniles were 195 and 49 μg mL^{-1} for HEX and ACET, respectively. Both acids significantly inhibited *M. javanica* undifferentiated egg hatch and J2 release from free eggs immersed in 100 μg mL^{-1} solutions, but only HEX achieved activity when egg masses were treated with acids' concentrations greater than 50 μg mL^{-1}. HEX lasted longer in soil than ACET did and yielded less females of *M. javanica* per gram of tomato root (EC_{50} = 112 mg kg^{-1}). Other than efficacy, the two acids had a negative impact on the free-living nematode abundances compared to the control, thus implying an eco-toxic character. MWE is effective for the target nematodes and increases the abundance of free-living nematodes and the microbial biomass.

Keywords Carboxylic acids · Soil communities · Chinaberry · Nematicidal

Key message

- Is it better to use crude extracts or purified ingredient compounds for root knot nematodes control?
- Acetic and hexanoic acids exhibit egg-hatch inhibition activity on embryonated eggs and J2 release.
- Acetic and hexanoic acids arrest *Meloidogyne javanica* biological cycle in tomato plants and are not phytotoxic.
- Acetic and hexanoic acids do not harm soil microbials.
- *Melia azedarach* extract, naturally containing acetic and hexanoic acids, increases abundancies of soil microbes and free-living nematodes.

Introduction

Meloidogyne javanica has been one of the three major root knot nematodes identified in Greece even before 1990 (Tzortzakakis et al. 2019). Plant-based bionematicides are in focus of research as eco-friendly alternatives to synthetic pesticides by reducing the undesired environmental impacts

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and side effects on human health. Progress is being made for their commercialization, illustrated by the number of biopesticides and related products in the registration pipeline, and major commercial opportunities exist for new bionematicides, in part occasioned by the phase-out of methyl bromide (Seiber et al. 2019). However, the effects of bionematicides on non-target organisms and the environment are not studied to the same extent as efficacy is. Biopesticides are considered as low-risk compounds, a belief mainly based on their natural origin rather than on experimental evidence. Thus, there is a need to explore the ecotoxicity of biopesticides and mostly their impact on soil microbes which is largely unknown (Rousidou et al. 2013). Similarly, the nematode free-living community is a useful indicator of changes in soil ecology attributed to improper fertilizer application, tillage, and pesticide application (Grabau et al. 2018). In every case, the influence of organic materials on the soil nematode community is associated with their chemical composition and so is its efficacy of controlling plant parasitic nematodes (Li et al. 2018). Thus, botanical matrixes used as powders or as crude extracts should be checked against their active ingredient components to discern for efficacy on target organisms as well as side effects on non-target ones.

Previously, we have reported that *M. azedarach* ripe fruits powder (MFP), tested in the soil at the rates of 30 and 60 g kg^{-1}, exhibited nematicidal activity similar to the one of fenamiphos (0.02 g a.i kg^{-1}) in terms of nematode population in roots and soil as well as reproduction rate (Cavoski et al. 2012). When the MFP was extracted with water to produce the ripe fruit water extract (MWE) applied at 1.75% w/w (expressed to extract dry yield) to treat *M. incognita*- and *M. javanica*-infested tomato plants, the efficacy levels were 68 and 80%, respectively (Ntalli et al. 2018). Most importantly, MFP and MWE, tested under actual field conditions, equally suppressed *Meloidogyne* spp. with the commercial nematicide oxamyl (Vydate® 10 SL) (Ntalli et al. 2018). In previous studies, we discerned hexanoic and acetic acids among MWE active ingredient components on *M. incognita* and we calculated their $EC_{50/1d}$ values at 38.3 and 41.1 µg mL^{-1}, respectively, by J2 paralysis evaluation (Ntalli et al. 2010). We also demonstrated that acetic acid harms the cuticle, degenerates the nuclei of pseudocoel cells, and vacuolizes the cytoplasm of *M. incognita* J2 (Ntalli et al. 2016).

The scope of this study was to assess the mode of action of hexanoic and acetic acids on nematode egg hatch and reproduction along with their fate in soil and side effects on soil microcosms. Herein, we report for the first time: (A) the efficacy of pure hexanoic (HEX) and acetic acid (ACET) on *M. javanica* in terms of (1) J2 paralysis activity; (2) egg-hatch inhibition properties after the treatment of (a) *free eggs* and successful assessment of impeded cell division in undifferentiated eggs and suppression of J2 exclosure as well as (b) *egg masses*; (3) biological cycle arrest in containerized culture of tomato and phytotoxicity evaluation; (B) the dissipation of the nematicidal acids in soil used in their pure form and as MWE components; (C) the effects of the acids on other (1) soil microorganisms using phospholipid fatty acids (PLFA) and (2) the abundance of free-living nematodes. We compared effects with those of the nematicidal MWE naturally containing the two acids.

Materials and methods

Nematodes rearing

Meloidogyne javanica originally sampled from naturally infested tomato greenhouses in Heraklion, Crete. Population was reared on tomato and freshly hatched (24 h) second-stage juveniles (J2), and different growth stages eggs were extracted from egg masses according to Hussey and Barker (1973) from 60-day nematode-infested roots. Egg masses were handpicked from roots using a stereoscope.

MWE preparation and chemicals

Ripe *M. azedarach* fruits were collected from trees in the campus of the Aristotle University of Thessaloniki, Greece. Voucher specimens were deposited in the Department of Ecology, School of Biology, Aristotle University of Thessaloniki, Greece. Fruits were crushed into fine particles to produce the MFP and extracted (150 g, 15-min sonication) with 218 mL water yielding 28.7 ± 0.4% w/v dry MWE. Acetic and hexanoic acids were purchased from Sigma-Aldrich. All solvents used for chemical analysis were HPLC grade.

Nematicidal bioassays on *M. javanica*

Paralysis activity of J2 treated with HEX and ACET

The paralysis activity of acetic and hexanoic acids was studied on *M. javanica* J2, and EC_{50} values were calculated according to Ntalli et al. 2010. Briefly, Cellstar 96-well plates (Greiner bio-one) were employed to immerse 20–25 J2 per well, in test solutions of 12.5 to 400 µg mL^{-1} of acetic acid and 50 to 800 µg mL^{-1} of hexanoic acid. Dose rates were established according to preliminary experiments. Paralysis was evaluated under an inverted microscope (Euromex, The Netherlands) at 40× after 1, 2, 3 and 4 days at 27 °C, after which nematodes were transferred in plain water to test for activity regain. Treatments were replicated five times, and each experiment was performed twice.

Egg-hatch inhibition of eggs treated with HEX and ACET

Activity on free eggs Procedures were according to Ntalli et al. (2013) and Oplos et al. (2018). Briefly eggs suspensions of mixed-development stages were mixed into 24-well cell culture plates (Greiner bio-one) with the test solutions at 50, 100 and 500 µg mL^{-1}. Cumulative time course of hatching data was performed by counting undifferentiated eggs and J2, since day 0 under an inverted microscope at 40×. Successive assessments were made after 4, 8 and 12 days. Cumulative percent J2 release was calculated using the formula:

$$\text{Cumulative percent J2 release} = \frac{\text{J2Dx} - \text{J2DO}}{\text{total}} \times 100$$

where Dx = day after the start of the assay. Cumulative percent undifferentiated egg hatch was calculated using the formula:

$$\text{Cumulative percent undifferentiated egg hatch} = \frac{\text{Eggs DO} - \text{Eggs Dx}}{\text{total}} \times 100$$

where Dx = day after the start of the assay. Five wells were used per treatment per assay, and two separate assays were performed in time.

Activity on eggs contained in egg masses The egg-hatch inhibition test on egg masses was performed according to Ntalli et al. (2013) and Oplos et al. (2018). Mature egg masses were treated with test solutions at 50, 100 and 500 µg mL^{-1}, in small plastic extracting trays made by 6-cm Petri dishes. Five days later, the test solutions were replaced with tap water and assessments of hatched J2 counts were performed every 7 days, when the water was replaced with fresh one. The experiment was completed when egg hatch stopped in the control treatment. The variable percentage of eggs that remain unhatched in the control was 8%. Consecutive assessment hatch counts in the control were added, until hatch arrest, and their sum was considered 100%. This value was used to correct the hatch values in experimental treatments. The experiment was performed twice, and every treatment was replicated five times.

Pot bioassays: efficacy, dissipation and ecotoxicity of HEX and ACET

Soil free of root knot nematodes was collected from the experimental farm of School of Agriculture, Aristotle University of Thessaloniki, and it was characterized as a clay loam with 1.3% organic matter and pH 7.8 according to Karpouzas et al. (2004) and Papadopoulou et al. (2011). Sequentially, it was mixed with sand at a ratio of 2:1 and was artificially inoculated with freshly hatched (2 days) *M. javanica* J2 in the concentration of 1000J2/100 g soil to be used for the efficacy experiments reported hereafter.

Estimation of EC_{50} values The EC_{50} values were estimated according to Ntalli et al. (2010) and Ntalli et al. (2018). The HEX and ACET were tested at the rates of 50 to 550 mg kg^{-1}. Water and carrier controls with ethanol were included in the experimental treatments. The experiments were performed in pots containing 200 g of soil each, where six-leaf stage tomato plants, cv. Belladonna were transplanted (one plant per pot). Plants were kept at 27 °C, 60% RH and 16-h photoperiod, and after the completion of a biological cycle (45 days), fresh root and shoot weight and total number of female nematodes per gram of root were assessed according to Ntalli et al. (2018). The experiment was replicated once, and the treatments were always arranged in a completely randomized design with five replicates.

Effect on soil microorganisms In order to evaluate the effect of HEX and ACET on soil microorganisms' community a pot bioassay was established as described by Ntalli et al. (2019). Five hundred grams of soil was used in 8×8×9 cm plastic pots each representing and treatment replicate. HEX and ACET were examined against the known nematicidal MWE all used at doses of expected activity over 60% efficacy. In particular, (1) MWE was tested at 1.7% w/w according to Ntalli et al. (2018), (2) ACET was tested at 650 mg kg^{-1} soil according to the single use pot bioassay reported herein, and (3) HEX was tested 250 mg kg^{-1} soil according to the single use pot bioassay reported herein. Water was used as an untreated control. A six-leaf stage tomato plant, cv. Belladonna, was transplanted in each pot, and 45 days later the fresh root and shoot weight along with total number of female nematodes per gram of root were assessed.

Furthermore, the effect of treatments on the parameters of the soil community was estimated. Specifically, the effects on soil microbial community assessed by phospholipid fatty acids (PLFAs) analysis. For analyses of PLFAs and free-living soil nematodes, two soil samplings were conducted: the first one early and the second in the end of the nematode biological cycle, i.e., 7 and 45 DAA. Extraction and analysis of phospholipids from soil samples was performed within 1 week of sampling, as reported by Ntalli et al. (2019), following the method described in detail by Papadopoulou et al. (2011). Overall, 27 fatty acid methyl esters were present in all samples and were further analyzed, including the internal standard 19:0. These fatty acids were assigned to functional

groups as follows (Findlay 2004; Papadopoulou et al. 2011; Ntalli et al. 2018, 2019): i-15:0, a-15:0, 15:0, i-16:0, i-17:0, 17:0 (Gram-positive bacteria; cy17:0, 16:1ω9c, 16:1ω9t (Gram-negative bacteria); 10Me16:0,10Me17:0, 10Me18:0 (actinomycetes); 18:2ω9,12 (fungi); 20:5ω3 and 20:3ω6 (protozoa); 20:0, 22:0, 23:0, and 24:0 (microeukaryotes, e.g., algae, nematodes). The remaining PLFAs may derive from several sources and were considered only for the estimation of total microbial biomass. For example, 18:1ω9t, 18:1ω9c may derive from both Gram-negative bacteria and fungi, 16:0 from bacteria and fungi, while 11:0, 13:0,14:0, 18:0, and 18:2ω6t are mainly of microbial origin. The sum of all identified lipid amounts was used as an index of total microbial biomass, and the ratios Gram+/Gram− and bacteria/fungi (B/F) were calculated.

Effects on soil saprophytic nematodes We used Cobb's sieving and decanting method as modified by S'Jacob and van Bezooijen (1984). The free-living nematodes were identified to genus level with the identification key of Bongers (1994). Nematode genera were assigned to trophic groups according to Yeates et al. (1993). The following microbial feeding genera were found in our experimental plots: the bacterivorous genera *Rhabditis, Mesorhabditis, Diploscapter, Heterocephalobus, Cervidellus, Acrobeloides, Eucephalobus, Boleodorus*, and *Panagrolaimus*; the fungivorous genera *Ditylenchus, Aphelenchus*, and *Aphelenchoides*. All other soil free-living nematodes, i.e., omnivores, predators, and non-parasitic plant feeders, were of very low abundance and therefore not presented in this study.

Fate of HEX and ACET in soil The soil was separated into four 1-kg samples which were spread evenly on plastic films in the laboratory and were treated with appropriate amounts (100 mL) of aqueous solutions of HEX and ACET, to receive final doses of 250 and 650 mg/kg, respectively. The third sample was treated with appropriate amounts of MWE, namely 17 g dry extract kg^{-1} soil. The selected test concentrations correspond to over 65% efficacy, according to previous experiments, and were used the same also for ecotoxicological experiments reported in the previous paragraph. In all cases, a total volume of 100 mL solution was applied for each treatment, in order to adjust the final water content to 40% of the maximum water-holding capacity (MWHC). Finally, the last sample was treated with the same amount of water to serve as control. All samples were briefly mixed by hand, and subsequently, bulk samples were divided into 21 subsamples (25 g), which were placed in aerated plastic bags (100 mL) and incubated in the dark at 20 °C. Immediately before incubation and 4 h, 19 h, 24 h, 48 h, 96 h, 168 h, and 14 days after treatment, triplicate samples from each treatment and soil were removed from the incubator and analyzed for residues as follows:

Soil samples (20 g) were extracted (1 h) with 0.1 N NaOH (40 mL) using an Orbi-shaker. After centrifugation (7000 rpm, 15 min), the supernant was acidified (1 M HCl) to pH = 2.5, filtered through a PTFE Q 0.22-µm filter and analyzed in HPLC system Spectra System TSP (Thermo Separation Products, Austin, TX, USA). A Zorbax Eclipse XDB-C18 column (4.6 mm × 250 mm, 5 µm) was used, the injection volume was 50 µl, and the flow rate of the mobile phase was 1 mL/min. Mixtures of acetonitrile and 10 mM KH_2PO_4 solution, pH 2.5, with different elution strengths were used as the mobile phase to analyze hexanoic and acetic acids, and detection was achieved at 210 nm. For quantitation, a seven-point calibration curve was constructed in the range 0.01–2.0 mg/mL (hexanoic and acetic acids) with good linearity ($R^2 = 0.9939$ and 0.9915). For method validation, recovery assays were conducted with soil samples fortified with HEX and ACET at three fortification levels (100, 500, and 1000 mg/kg) and three replicates for each level. Recoveries ranged between 91.0–112% and 95.4–109% for HEX and ACET, respectively. Extracts of soil samples treated with MWE were also analyzed for HEX and ACET in order to follow the fate of MWE in soil.

Statistical analysis

For all efficacy bioassays, since ANOVA indicated no significant treatment by time interaction, means were averaged over runs of the experiments. The percentages of paralyzed J2 observed in the microwell assays were corrected by removing the natural death/paralysis in control according to the Schneider–Orelli's formula (Puntener 1981):

$$\text{Corrected } \% = \frac{\%\text{ mortality in treatment} - \%\text{ mortality in control}}{100 - \%\text{ mortality in control}} \times 100$$

and they were analyzed (ANOVA) after being combined over time. Corrected percentages of paralyzed J2 treated with test solutions were subjected to nonlinear regression analysis using the log-logistic equation proposed by Seefeldt et al. (1995):

$$Y = C + \frac{D - C}{1 + \exp\left[b\left(\log(x) - \log(\mathrm{EC}_{50})\right)\right]}$$

where C = the lower limit, D = the upper limit, b = the slope at the EC_{50}, and EC_{50} = the test compound concentration

required for 50% death/paralysis of nematodes after eliminating the control (natural death/paralysis). In the regression equation, the test concentration was the independent variable (x) and the paralyzed J2 (percentage increase over water control) was the dependent variable (y). The mean value of the five replicates per test concentration and immersion period was used to calculate the EC_{50} value. The 95% confidence intervals (CI 95%) were determined for toxicity comparison.

For egg-hatch inhibition bioassays, both concerning free and contained in egg masses eggs, treatments means were compared using Tukey's test at $P \leq 0.05$. Data of J2 released from egg masses were expressed as a percentage decrease in the number of J2 released from control, according to the Abbott's formula:

$$\text{Corrected } \% = 100 \times \left(1 - \frac{\text{J2 in treated plot}}{\text{J2 in control plot}}\right)$$

The data from the pot experiments were expressed as a percentage decrease in the number of females or galls per gram of root corrected according to the control, using the Abbott's formula:

$$\text{Corrected } \% = 100 \times \left(1 - \frac{\text{females number in treated plot}}{\text{female number in control plot}}\right)$$

It was fitted in the log-logistic model (Seefeldt et al. 1995) to estimate the concentration that caused a 50% decrease in females and galls per gram of root (EC_{50} value). In this regression equation, the test compound was the independent variable (x) and the female nematodes, percentage decrease over water control, were the dependent variable (y). For efficacy pot bioassay performed with single acids against MWE, treatments means were compared using Tukey's test at $P \leq 0.05$.

Repeated-measures ANOVA was performed on soil free-living nematodes and PLFAs data to determine the effect of sampling time, treatment and their interaction. In all analyses, means were compared using Tukey's test at $P < 0.05$. Before analyses, data were transformed appropriately when necessary in order to meet the assumptions of ANOVA.

Results

Paralysis experiments on J2

When HEX and ACET were tested individually on *M. javanica* J2, they achieved paralysis in a dose- and time-dependent manner (Table 1). HEX was found more active than ACET. In particular, the achieved EC_{50} values for HEX ranged from 339 to 195 µg mL^{-1} after 1 to 4 days of J2 immersion in test solutions, while for ACET the respective EC_{50} values were 162 to 49 µg mL^{-1}. In all cases, J2 never regained motility after moving to water and paralysis was irreversible.

Table 1 EC_{50} values (µg mL^{-1}) of Hexanoic acid-HEX and Acetic acid-ACET paralysis activity on *Meloidogyne javanica* after 1, 2, 3 and 4 days of nematodes' immersion in test solutions with respective standard error and confidence interval values

	EC_{50} (µg mL^{-1})	SE	95% CI
HEX			
1 day	339.3	14.260	309.8–368.8
2 days	233.0	11.820	208.5–257.4
3 days	208.4	9.992	187.8–229.1
4 days	195.8	11.520	172.0–219.7
ACET			
1 day	162.4	6.806	148.3–176.7
2 days	85.8	6.401	72.6–99.0
3 days	55.0	2.880	49.1–60.9
4 days	49.0	2.986	43.7–56.1

Egg-hatch inhibition on free eggs treated with HEX and ACET

Both HEX and ACET significantly decreased the cumulative egg hatch only for undifferentiated eggs immersed in 100 and 500 µg mL^{-1} solution (Fig. 1a, b). HEX lessened significantly since 4 days the cumulative undifferentiated egg hatch when used at 500 µg mL^{-1}, while the test concentration of 100 µg/mL exhibited slower action significantly differing from control values 8 days post-experiment establishment (Fig. 1a). The cumulative undifferentiated egg hatch decreased significantly for ACET test concentrations from 100 and 500 µg mL^{-1} (Fig. 1b). Specifically, 4 days post-experiment establishment the undifferentiated egg hatch for the control was 31 ± 5.0, while for the acetic acid used at 100 and 500 µg mL^{-1} it was 11 ± 3.5 and 10 ± 3.0, respectively.

Concerning J2 release, HEX exhibited again a slow action since at the first assessment date only the highest test concentration considerably differed from control values. In the two successive assessment dates, that is, 8 and 12 days post-experiments start all test concentrations yielded significantly lower J2 considering control values, while eggs treated with 500 µg mL^{-1} almost arrested J2 release since 8 days post-experiment start (Fig. 2a). Eight days post-experiment establishment, ACET caused a considerable decrease of J2 release of *M. javanica* when tested at the concentration of 100 and 500 µg mL^{-1} (Fig. 2b). Interestingly, 4 days post-eggs immersion in test solutions the percent J2 release at the treatment

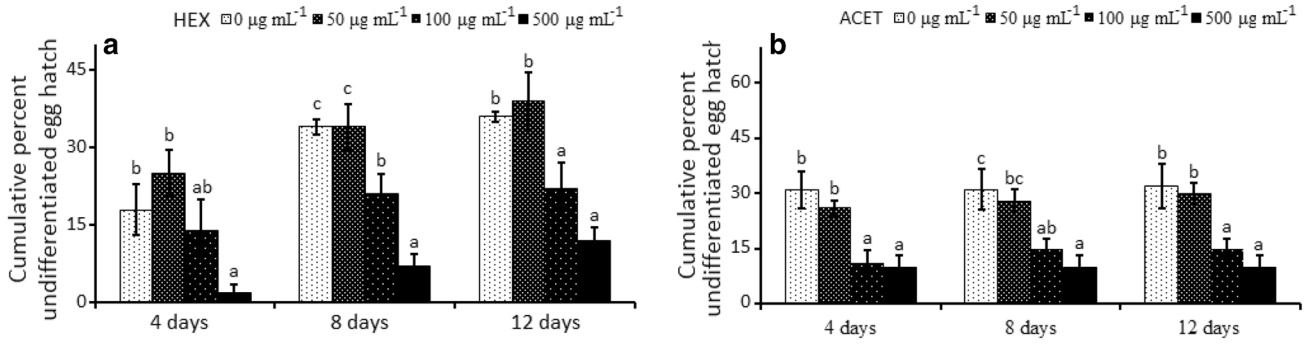

Fig. 1 Effect of hexanoic acid—HEX (**a**)—and acetic acid—ACET (**b**)—on cumulative percent hatch of *Meloidogyne javanica* undifferentiated eggs 4, 8, and 12 days post-experiment establishment. Data represent the mean ± SD from two experiments performed in time, with 5 replicates per treatment each. Values within each assessment date were compared using Tukey's test, and those followed by different letters are significantly different at ($P \leq 0.05$)

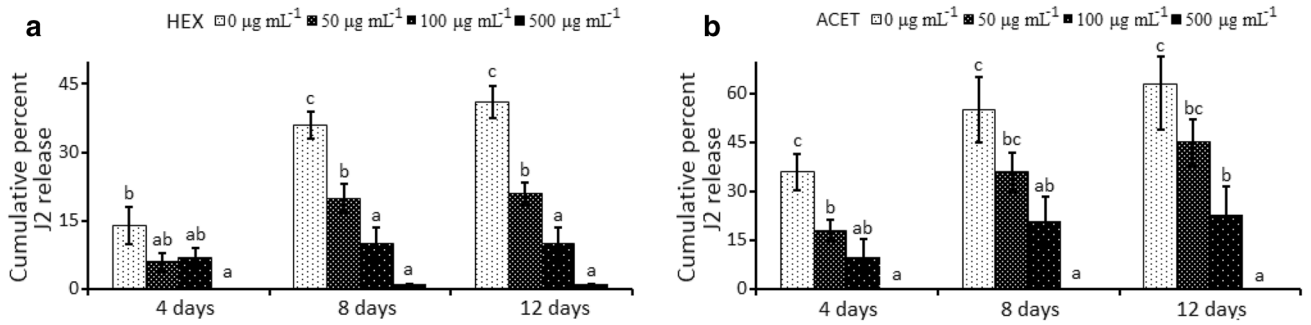

Fig. 2 Effect of hexanoic acid—HEX (**a**)—and acetic acid—ACET (**b**)—on cumulative percent release of *Meloidogyne javanica* J2 4, 8, and 12 days post-experiment establishment. Data represent the mean ± SD from two experiments performed in time, with 5 replicates per treatment each. Values within each assessment date were compared using Tukey's test, and those followed by different letters are significantly different at ($P \leq 0.05$)

of 50 µg mL^{-1} was 18 ± 3.5, thus significantly lower to the control (36 ± 5.5%). In the assessments made 8 and 12 days post-experiment establishment, the percent J2 release at 100 µg mL^{-1} was 21 ± 7.5 and 23 ± 8.5, statistically different from the control counting 55 ± 10 and 63 ± 14 (Fig. 2b). For what concerns the test concentration of 500 µg mL^{-1}, it completely arrested J2 release since day 2.

Egg-hatch inhibition in egg masses treated with HEX and ACET

The hatch inhibition was evident since day 7 for egg masses immersed in test solutions of all HEX test concentrations (Fig. 3). In 21 and 28 assessment days, the percent total hatch in control was only 13 and 7% thus not allowing differences regarding efficacy of treatments. Only the smallest test concentration exhibited a relatively slow action, differing from control since day 8 onward. On the contrary, ACET used at the same test concentration did never exhibit

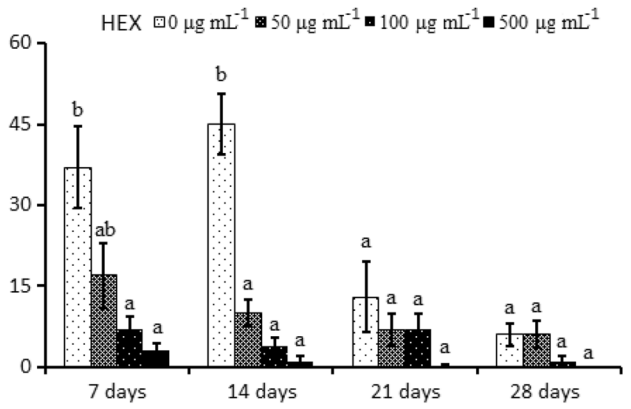

Fig. 3 Effect of hexanoic acid–HEX—on *Meloidogyne javanica* hatch, after immersion of egg masses at the dose rates of 10, 50, and 500 µg mL^{-1} for 5 days. At the time of the assessment, the egg hatch at the control treatment was at 8% over total corresponding to the maximum recorded on week intervals. Values within each assessment date were compared using Tukey's test, and those followed by different letters are significantly different at ($P \leq 0.05$)

egg-hatch inhibition activity statistically different to the control (data not presented).

Biological cycle arrest and EC_{50} calculation of HEX and ACET

When the carboxylic acids were used to treat soil artificially inoculated with nematodes, HEX exhibited the highest activity and the calculated EC_{50} value was 112.4 mg kg^{-1} based on female counts per gram of infested root (Table 2). ACET followed with an EC_{50} value of 440.6 mg kg^{-1} based on female counts per gram of infested root. The same efficacy trend was established for the two acids according to gall counts per gram of tomato roots. Most interestingly, none of the two acids inhibited plant growth or provoked phytotoxicity to tomato plants even when used at the highest test concentrations of 550 mg kg^{-1}.

When the acids were used against MWE in a pot experiment at test concentrations of expected efficacy over 60%, all treatments significantly differed to control. MWE counted less females and galls per gram of root than the acids did (Fig. 4), and HEX yielded significantly less galls than ACET. No effect was evident for what concerns aerial and root weights.

Effects of HEX and ACET on soil microbial community and free-living nematodes

All treatments decreased significantly the number of *M. javanica* J2 juveniles per 100 mL of soil on 40th day (Fig. 5). The numbers of J2 in soil on 7th day were trivial and thus not presented. HEX and ACET were the most effective treatments, while the application of MWE resulted in higher J2 numbers per 100 mL of soil.

Repeated-measures ANOVA revealed that all microbial groups were affected by sampling time and treatments (Fig. 6). On 7th day, no significant differences were recorded among the experimental treatments regarding Gram-positive bacteria, actinomycetes, fungi, and protozoa biomass. The biomass of Gram-negative bacteria was increased by the

Table 2 EC_{50} values (mg kg^{-1} soil) of Hexanoic acid-HEX and Acetic acid-ACET efficacy on *Meloidogyne javanica* as calculated in pot experiments with respective standard error and confidence interval values

	EC_{50} (mg kg^{-1})	SE	95% CI
HEX			
♀/g root	112.464	20.769	69.50–155.428
Galls/g root	108.230	17.587	71.848–144.611
ACET			
♀/g root	440.669	39.655	358.636–522.702
Galls/g root	451.691	41.811	365.198–538.183

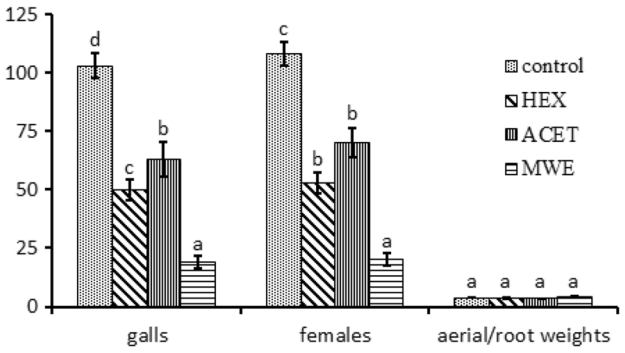

Fig. 4 Tomato (*Solanum lycopersicum*) root infestation with *M. javanica* (galls and females per gram of root) and respective growth parameters, after treating with hexanoic acid—HEX (250 mg kg^{-1}), acetic acid—ACET (650 mg kg^{-1}), and Melia water extract—MWE (1.7% w/w). Different letters above columns correspond to statistically significant differences between treatments (one-way ANOVA, Tukey's test at $P < 0.05$)

MWE treatment, while the numbers of microeukaryotes were decreased by the application of HEX and ACET. On 40th day, we have a different pattern, as all estimated microbial groups exhibited significantly higher biomass values in the MWE treatment. HEX and ACET samples presented similar values to the control, with the exception of microeukaryotes presenting significantly decreased biomass values as it was recorded on 7th day. The ratio Gram+/Gram− presented the same pattern in both samplings, as MWE application was found to decrease it compared to the control and the other treatments. The ratio bacteria/fungi, on the other hand, was not affected by treatment and presented higher values in the second sampling.

Regarding the microbivorous free-living nematodes, both bacterial and fungal feeders presented a similar pattern, in both samplings. On the 7th day, the application of HEX and ACET had a very strong negative impact on the populations

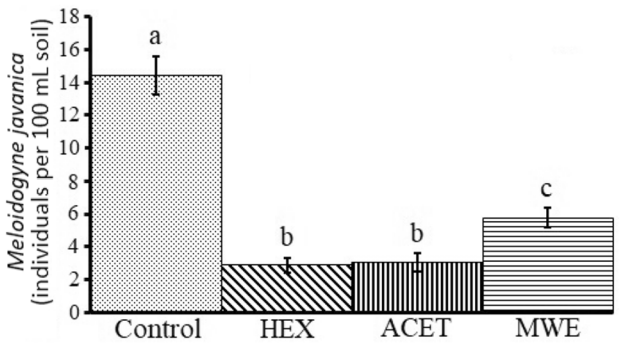

Fig. 5 Effect of treatments against *Meloidogyne* sp. (J2/100 mL soil ± SE) 40 days after application. Different letters above columns correspond to statistically significant differences between treatments (one-way ANOVA, Tukey's test at $P < 0.05$)

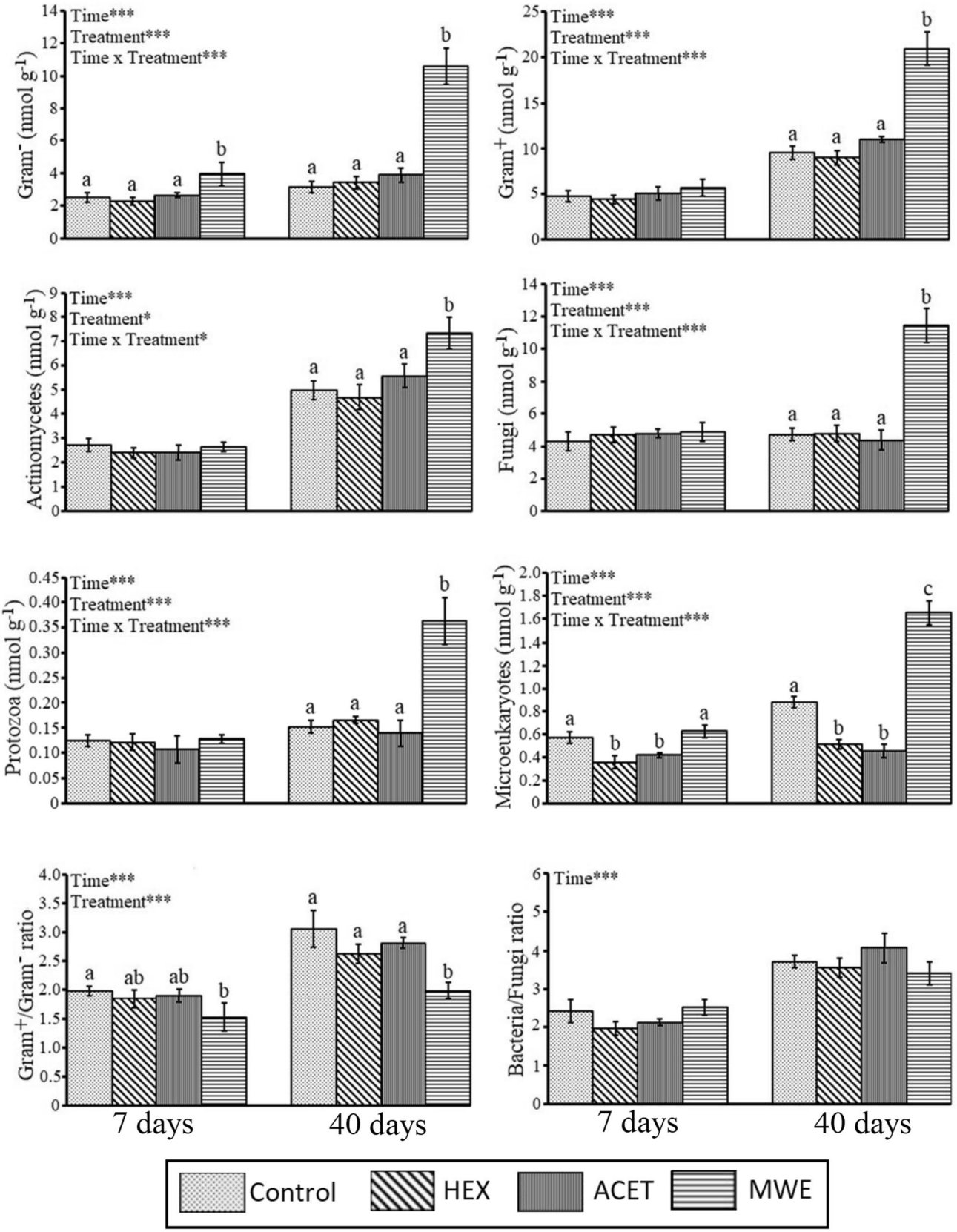

Fig. 6 Mean biomass (±SE) of different microbial groups and PLFA ratios recorded 7 and 40 days after application. The results of repeated-measures ANOVA are indicated on each graph (*$P < 0.05$, ***$P < 0.001$), while different letters above columns correspond to statistically significant differences between treatments within each sampling event (Tukey's test)

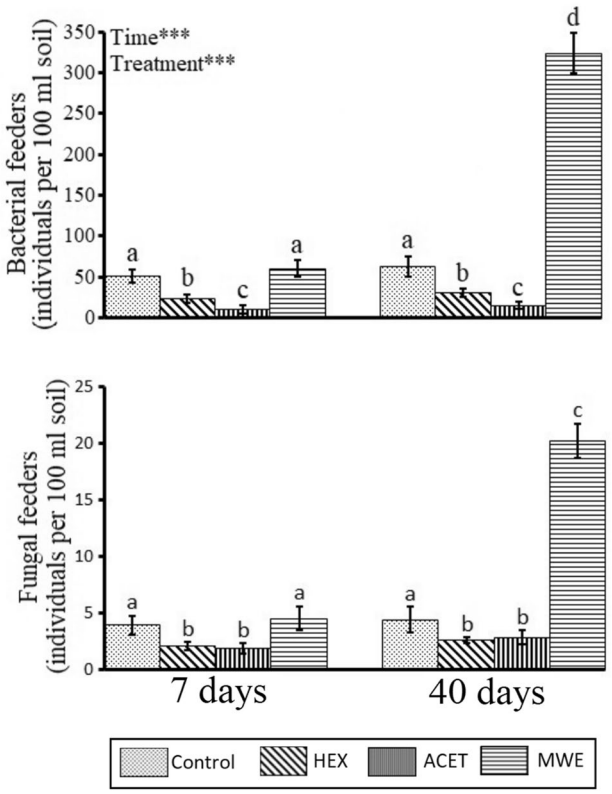

Fig. 7 Mean abundance (±SE) of bacterivorous and fungivorous nematodes recorded 7 and 40 days after application. The results of repeated-measures ANOVA are indicated on each graph (***$P < 0.001$), while different letters above columns correspond to statistically significant differences between treatments within each sampling event, as indicated by Tukey's test

Table 3 The half-life $T_{1/2}$ values of Hexanoic acid-HEX and Acetic acid-ACET in soil samples together with the first-order degradation rate K_{deg} and r^2

	$T_{1/2}$ (h)	K_{deg} (1/h)	R^2
HEX	15.1	0.046	0.9953
ACET	18.7	0.037	0.9206

of bacterial feeders and fungal feeders; the ACET treatment decreased the bacterivorous nematodes much more than the HEX. On the 40th day, the HEX and ACET treatments still presented lower free-living nematode abundances compared to the control; on the contrary, the MWE treatment enhanced the population of both bacterivorous and fungivorous nematodes (Fig. 7).

Dissipation of HEX and ACET in soil

Degradation of HEX and ACET in soils followed first-order kinetics, and their $T_{1/2}$ was calculated using the following formula: $T_{1/2} = \ln 2/K_{deg}$ (1) where $T_{1/2}$ (h) is the pesticide half-life and K_{deg} is the pesticide first-order degradation rate (1/h). The $T_{1/2}$ and K_{deg} of HEX and ACET in soil samples tested are summarized in Table 3. $T_{1/2}$ for HEX and ACET ($P < 0.05$) was estimated to be 15.1 and 18.7 h, respectively.

Discussion

In the frame of studying the mode of action of nematicidal molecules herein, we report on the multistage inhibition of HEX and ACET on *M. javanica* for the first time. The $EC_{50/4d}$ values calculated for paralysis activity of HEX and ACET on *M. javanica* were calculated at 195 and 49 µg mL^{-1}, respectively. It seems that *M. javanica* is not as sensible to ACET as the species *M. incognita* for which the $EC_{50/1d}$ value was 38 µg mL^{-1} as reported in our previous works (Ntalli et al. 2010). In particular, earlier we proved that the ACET harms the cuticle, degenerates the nuclei of pseudocoel cells, and vacuolizes the cytoplasm of *M. incognita* (Ntalli et al. 2016). When *M. javanica* eggs of different embryonic stages were exposed to HEX and ACET for 4, 8, or 12 days both acids used at 100 µg mL^{-1} or more, the cumulative undifferentiated egg hatch was significantly decreased. This means that the eggs containing one cell remained at this stage till the end of the experiment and were never subjected to cell division. Similarly, eggs immersed at test concentrations greater than 100 µg mL^{-1} hatched to J2 significantly less considering control values. Interestingly, when egg masses were immersed in test solutions instead of free eggs, only HEX was effective in arresting the hatch, while ACET did not differ to control counts (data not shown). This might have to do with the different physicochemical properties like lipophilicity and eventual different egg mass penetration ability of the two acids (Ntalli et al. 2016). In pot bioassays, HEX arrested *M. javanica* cycle in tomato roots better than ACET, and the calculated EC_{50} values were 112 and 440 mg kg^{-1}, respectively. Since $T_{1/2}$ values for the two acids are similar, the better efficacy of HEX cannot be attributed to faster degradation in soil and other factors should be studied like its activity on nematode growth stages developing in host roots. According to the above-mentioned efficacy values, HEX and ACET seem promising tools in nematodes' management, necessitated after the ban of many synthetic pesticides due to human and environmental threats (CD 91/414/EE and EC 1107/2009). In fact, many plant metabolites are now examined as alternative tools in the frame of an integrated nematode management, but results focus mainly on efficacy matters of extracts and active ingredients (Hernández-Carlos and Gamboa-Angulo 2019; Ntalli and Caboni 2012; Andrés et al. 2012) and formulation issues (Borges et al. 2018). Among botanical pesticides, only some commercial ones were studied for their effects on soil microbial functions (Suciu et al. 2019; Ipsilantis et al. 2012; Spyrou

et al. 2009). But nematicides by nature can have a detrimental effect in soil functionality, since they are directly applied in it; thus, plant secondary metabolites and botanical extracts need to be studied also in that direction along with efficacy evaluation. In our previous study, we proved that furfural, representing a most potent fumigant component of *Melia azedarach*, adversely affected the soil community and especially the free-living nematodes (Ntalli et al. 2018). Herein, we test the already proven nematicidal botanical extract MWE against the two acids used individually, all in doses of expected efficacy over 60%, to study the side effects on soil microbial communities and free-living nematodes. We proved that regarding the microbivorous free-living nematodes, both bacterial and fungal feeders presented a similar pattern, in both samplings. On the 7th day, the application of HEX and ACET had a very strong negative impact on the populations of bacterial feeders and fungal feeders; the ACET treatment decreased the bacterivorous nematodes much more than the HEX. On the 40th day, the HEX and ACET treatments still presented lower free-living nematode abundances compared to the control. Interestingly, the two acids did not harm any of the soil microbial groups revealing a more eco-friendly character than furfural had in our previous studies (Ntalli et al. 2018). Most importantly, MWE enhanced the population of both bacterivorous and fungivorous nematodes. The decreased numbers of microeukaryotes recorded in the HEX and ACET treatments are in accordance with the lower nematode abundances in the same soil samples. Microeukaryotes biomass expresses the number of algae, nematodes, etc., found in the soil and was expected to be significantly decreased as the two acids have a drastic negative effect on soil free-living nematodes. Also previously, we reported that MWE enhanced the proliferation of soil microbes and microbial feeding nematodes compared to its constituent nematicidal components used individually (Ntalli et al. 2018).

According to the PBT assessment, HEX (CAS 142-62-1) is a biocide that is readily biodegradable within 28 days and is not considered as a toxic substance regarding ecotoxicological endpoints (www.ECHA.com), while ACET is included in Annex I of Directive 91/414/EEC as a herbicide (SANCO/2602/08-rev. 5; Commission Implementing Regulation No 540/2011). Since HEX and ACET exhibit also a multistage activity against *M. javanica* and are not toxic to tomato plants, they could be of possible consideration also as actives for nematicidal plant protection products. As for the nematicidal MWE even promoting soil microorganisms and free-living nematodes, it could be of practical use for developing countries or/and as a self-made and ready-to-use supplementary nematode control tool. Of course, a short evaluation under the basic substances' regulation should be anticipated (SANCO/ 10363/2012 rev.9).

Author contributions

NN, UM-S, and NM conceived and designed the research; NN and MN analyzed data; NN and NM wrote the manuscript; KD, MK, E-NP, GB, and MD conducted experiments; NN, U M-S, and NM supervised biological experiments and provided critical corrections of the manuscript; all authors read and approved the manuscript.

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Compliance of ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

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