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ORIGINAL PAPER

Nematotoxic activity of essential oils from *Monarda* species

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Abstract
Bioactivity of essential oils (EOs) from *Monarda* species has never been investigated on phytoparasitic nematodes. In this study, the EOs from two Italian ecotypes of *Monarda didyma* and *M. fistulosa* and their main compounds, carvacrol, γ-terpinene, *o*-cymene, and thymol, were evaluated for their in vitro activity on the infective stages of phytoparasitic nematodes *Meloidogyne incognita* and *Pratylenchus vulnus*, as well as on *M. incognita* egg hatch. Soil treatments with the two EOs were also investigated for their suppressiveness on *M. incognita* on tomato. Both EOs were strongly active on *M. incognita* juveniles, as a only 1.0 µL mL^{-1} LC50 value was evaluated after a 24-h exposure to both EOs, whereas a lower activity was recorded on *P. vulnus* (15.7 and 12.5 µL mL^{-1} LC50 values for *M. didyma* and *M. fistulosa* EOs, respectively). Among the EOs' main compounds, carvacrol was highly active also at a short exposure in low concentrations, whereas γ-terpinene and thymol were much less active on both nematode species and *o*-cymene showed a discrete activity on *P. vulnus* only at the highest concentration. Hatch percent of *M. incognita* eggs treated with *M. didyma* and *M. fistulosa* EOs was always significantly lower than in water or in Tween 20 and Oxamyl solutions. In the experiment in soil, the multiplication of *M. incognita* and gall formation on tomato roots was significantly reduced by soil treatments with both EOs. The strong nematicidal activity of both *Monarda* EOs may suggest them as potential sources of new sustainable nematicidal products.

Keywords *Monarda didyma* · *Monarda fistulosa* · Essential oils · Carvacrol · γ-Terpinene · *o*-Cymene · Thymol · *Meloidogyne incognita* · *Pratylenchus vulnus* · Nematicidal activity

Key message

- There is no information on the nematicidal activity of essential oils from *Monarda* species and on the relationship between nematoxicity and oil compositional profile.
- Essential oils from *Monarda* plants are strongly active on *M. incognita* juveniles and reduce nematode multiplication and gall formation on tomato roots.
- Essential oils from *Monarda* plants have a high potential for the development of new nematicidal products.

Introduction

Essential oils (EOs) are complex mixtures of bioactive volatile compounds, consisting of terpenes and terpenoids, oxygenated terpenes, sesquiterpenes and phenylpropanoids, produced by the secondary metabolism of aromatic plants such as Lamiaceae, Myrtaceae, and Umbelliferae (Bakkali et al. 2008).

Several biological activities have been reported for plant EOs and their main components, including also a biocidal activity against plant soilborne pathogens and pests, which also includes phytoparasitic nematodes (Isman 2000; Isman et al. 2011; Laquale et al. 2015; Avato et al. 2017).

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Phytoparasitic nematodes are harmful agricultural pests, annually causing heavy yield losses of a wide range of crops (Perry and Moens 2011). Most of the losses are due to root-knot nematodes of genus *Meloidogyne* (Wesemael et al. 2010), though serious problems are also caused by the root lesion nematode *Pratylenchus vulnus* Allen and Jensen, as the most important nematode of stone fruits in Mediterranean climate (Pinochet et al. 1991).

Concerns for environmental risks raised by soil treatments with synthetic nematicides need their replacement with more sustainable control tools (Lewis et al. 1997). Biocidal compounds produced by the secondary metabolism of a wide range of plants, among which also are essential oils and their components, have been one of the focal points of these alternative control strategies (Argentieri et al. 2008; Avato et al. 2013; D'Addabbo et al. 2013; Laquale et al. 2015).

Toxic effects of EOs against phytoparasite nematodes have been investigated mainly on root-knot nematodes and on the pine wilt nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, whereas data on EOs' activity on other phytonematode species are much fewer (Andrés et al. 2013; Laquale et al. 2015; Faria Silva et al. 2016; Avato et al. 2017). Activity on *M. incognita* (Kofoid et White) Chitwood and *M. javanica* Treub was extensively reported both in vitro and in soil experiments for the EOs from a large number of aromatic and medicinal plants and their terpene components (Oka et al. 2000; Ntalli and Menkissoglu-Spiroudi 2011; Ntalli et al. 2013; Laquale et al. 2015; Avato et al. 2017). Adversely, poor data are available on EOs' activity on *Pratylenchus* species (Tsao and Yu 2000; Avato et al. 2017).

The genus *Monarda* L. (*Lamiaceae*) includes about eighteen species of herbaceous, annual, or perennial plants endemic to North America (Duke 2002). Economic relevance of these plant species is related to the presence of EOs with highly active compounds as geraniol, linalool, thymol, and carvacrol (Mazza et al. 1993). In particular, a high content of geraniol was previously reported for the EO of wild bergamot *M. fistulosa* (Mazza et al. 1987), whereas high percentages of thymol and carvacrol were detected in the EO of *M. didyma* L. (golden balm) (Fraternale et al. 2006; Gwinn et al. 2010; Ricci et al. 2017).

Bioactivity of the EOs from *M. didyma* and *M. fistulosa* has been documented mainly on plant fungal pathogens, such as *Rhizoctonia solani* K. (Fraternale et al. 2006; Gwinn et al. 2010), *Sclerotinia sclerotiorum* de Bary (Gwinn et al. 2006) and *Botrytis cinerea* (Lib.) (Adebayo et al. 2013). To the best of our knowledge, there are no reports on the effects of the EOs from *Monarda* plants on phytoparasitic nematodes, but the strong nematicidal activity documented for the main monoterpene components of the EOs from *M. didyma* and *M. fistulosa* may suggest a high nematicidal potential of these EOs (Oka et al. 2000; Echeverrigaray et al. 2010; Ntalli et al. 2010). Therefore, this study was aimed to investigate the in vitro activity of two EOs from *M. didyma* and *M. fistulosa* on the infective stages of two phytonematode species with a different feeding and reproductive habitus such as *M. incognita* and *P. vulnus*, as well as on the hatch behavior of *M. incognita* eggs. The effectiveness of soil treatments with these two *Monarda* EOs was also investigated against *M. incognita* on tomato (*Solanum lycopersicum* L.) in potted mixes.

Materials and methods

Essential oils

Plants of *M. didyma* and *M. fistulosa* were cultivated at Giardino delle Erbe Officinali (Casola Valsenio, Ravenna, Italy). Specimens of each plant species are deposited at Department of Agricultural Sciences of University of Bologna (Bologna, Italy). Plant aerial parts were collected at middle October, and EOs were extracted by 2-h hydrodistillation using a Clevenger-type apparatus. EOs' yields (v/w) were 2.61 and 3.68 mL kg^{-1} plant biomass for plants of *M. didyma* and *M. fistulosa*, respectively. Oil distillates were dried overnight over anhydrous Na_2SO_4 and then kept in the refrigerator until analyzed.

Nematodes

Egg masses of an Italian population of *M. incognita*, previously reared on tomato *cv*. Roma in a glasshouse at 25 ± 2 °C, were handpicked from infested roots and then incubated in distilled water in a growth chamber at 25 °C. Emerged juveniles (*J2*) were collected and stored at 5 °C until used. Mixed-age infective specimens of *P. vulnus* were recovered from carrot disk culture (Moody et al. 1973) by washing with sterile distilled water and used immediately.

Compositional analysis of essential oils

A Trace GC–FID ultra Thermo Finnigan gas chromatograph was used for the chemical analysis of the above EOs. A sample (1 µL) of each distilled EO solubilized in hexane was injected in the cold on-column mode in a DB-5 (J&W Scientific) fused silica capillary column of 30 m × 0.25 mm; 0.25 µm film thickness. Chromatographic conditions were as follows: detector temperature 300 °C; column temperature was programmed from 60 °C (5 min isothermal) to 280 °C (30 min isothermal) at 4 °C min^{-1}. Hydrogen was the carrier gas (35 kPa; 2.0 mL min^{-1}); air and H_2 were adjusted to yield optimum separation; data were processed using the Chrom-Card 32-bit computing software. Quantitative data

of the EOs constituents were expressed as a percentage composition from the total peak areas detected from GC–FID analyses without the use of correction factors.

GC–MS analyses were performed with a Hewlett Packard 6890-5973 mass spectrometer interfaced with a HP Chemstation. Chromatographic conditions were as follows: column oven program from 60 °C (5 min isothermal) to 240 °C (15 min isothermal) at 3 °C min^{-1}; injector, 280 °C. Helium was the carrier gas (flow rate, 1 mL min^{-1}). A HP-5 MS capillary column (30 m × 0.25 mm; 0.25 µm film thickness) was utilized. MS operating parameters were: ion source, 70 eV; ion source temperature, 200 °C. Mass spectra were acquired over 40–800 amu range at 1 scan s^{-1}. The ion source was operating in the electron impact mode. Samples (1 µL) were injected using the splitless sampling technique.

Determination of the chemical composition of the analysed EOs was based on comparison of GC retention times of their constituents with authentic reference compounds in combinations with arithmetic indexes (AI) and by means of reference mass spectra from standard compounds and/or from NIST mass spectral library files (Avato et al. 2005; Adams 2007).

Nematode mortality bioassay

Batches of 100 *M. incognita J2* or *P. vulnus* infective specimens suspended in 0.5 mL distilled water were placed in 1.5 mL Eppendorf tubes. Appropriate amounts of the EOs from *M. didyma* and *M. fistulosa* or carvacrol, *o*-cymene, γ-terpinene, and thymol (purchased by Sigma-Aldrich, Italy), previously determined as their main components by GC and GC–MS, were added to a 0.3% Tween 20 water solution, as to prepare 6.25, 12.5, 25, 100, and 200 µg mL^{-1} solutions. A 0.5 mL volume of each solution was poured into each Eppendorf tube containing the nematode suspensions, as to obtain 3.12, 6.25, 12.5, 25, 50, and 100 µg mL^{-1} final test concentrations. Paired mixtures (2:1; 1:1 and 1:2) of *o*-cymene: carvacrol, γ-terpinene: carvacrol, and γ-terpinene: *o*-cymene in a 0.3% water solution of Tween 20 were also assayed at 6.25, 12.5, and 25 µg mL^{-1} concentrations.

Nematodes were exposed to each concentration of all test solutions for 4, 8, or 24 h. In all experiments, four replicates were provided for each concentration × exposure time combination, including distilled water, a 2 mL L^{-1} water solution of the nematicide Oxamyl (10% a.i.) and 0.3% Tween 20 as controls. After each exposure time, nematodes from each replicate were observed under a light microscope and ranked as motile or paralyzed. After each observation as above, nematodes of the two species were recovered on a 5-µm sieve and then transferred to distilled water. Nematode mortality was confirmed by the persistence of immobility at 72 h after placement in distilled water. Mortality rates were calculated according to Abbott's formula (Finney 1978) $m = 100 \times (1 - nt/nc)$, in which: *m*, percent mortality; *nt*, number of viable nematodes after the treatment; *nc*, number of viable nematodes in the water control.

Egg hatching bioassay

Batches of 50 egg masses of *M. incognita* (averaging 450 eggs per mass) from the same population used for mortality assays were placed in 1.5 mL Eppendorf tubes containing 0.5 mL of distilled water. The EOs of *M. didyma* and *M. fistulosa* or carvacrol, *o*-cymene, γ-terpinene, and thymol were added to a 0.3% Tween 20 water solution to prepare 1000 or 2000 µg mL^{-1} solutions. A 0.5 mL volume of these solutions was added to each Eppendorf tube containing the egg masses as to obtain final 500 and 1000 µg mL^{-1} concentrations. The egg masses were exposed to each concentration of the test solutions for 24 or 48 h. Four replicates were provided for each concentration × exposure time combination, including distilled water and a 0.3% Tween 20 water solution as controls. At the end of each exposure time, the egg masses were rinsed in distilled water and placed in 2-cm-diameter sieves (215 µm aperture). Each sieve was placed in a 3.5-cm-diameter Petri dish, and 3 mL of distilled water was added to submerge the egg masses. The Petri dishes were then arranged in a growth chamber at 25 °C throughout the hatching test duration.

Emerged *J2* were removed and counted at weekly intervals, repeatedly washing egg masses with sterile water and renewing distilled water at the same time. A non-destructive observation for the occurrence of microbial contamination on egg masses, as potentially affecting egg hatching, was done under an optical microscope after each weekly removal of juveniles.

The egg masses were removed from the sieves after a total of 5 weeks, and unhatched eggs were counted after dissolving the egg masses by a 3-min shaking in a 1% sodium hypochlorite aqueous solution (Hussey and Barker 1973). Total number of *J2* emerged during the hatching test was expressed as cumulative percentages of the total (hatched + unhatched) egg content of egg masses.

Experiment in soil

Roots of tomato plants infested with the same population of *M. incognita* used for the in vitro experiments were finely chopped, and the number of eggs and *J2* was determined by processing six 10-g root samples with a 1% aqueous solution of sodium hypochlorite (Hussey and Barker 1973). Appropriate amounts of this root inoculum were thoroughly mixed with a steam sterilized sandy soil (64.4% sand, 18.7% silt, 16.9% clay, 0.8% organic matter, and 7.5 pH), as to obtain an initial population density of 20 eggs and juveniles per mL^{-1}

soil. The infested soil was poured into 1-L clay pots arranged on benches in a greenhouse. Soil of each pot was then treated with 62.5, 125, 250 500, or 1000 µg kg^{-1} soil rates of *M. didyma* or *M. fistulosa* EOs vehiculated in 400 mL volume of a 0.3% water solution of Tween 20. Non-treated soil, either non-infested or infested by *M. incognita,* and soil treated with a 2 mL kg^{-1} soil rate of liquid Oxamyl, applied three days before transplanting, were used as controls. Five replicates per each treatment were provided, according to a randomized block design. A 1-month-old seedling of tomato cv. Roma was transplanted in each pot three weeks after treatments with EOs.

Plants were uprooted after 2-month maintenance at a 25 ± 2 °C constant temperature in the greenhouse. The fresh weight of aerial parts and roots of each plant was recorded, and galling caused by *M. incognita* was evaluated on each tomato root according to a 0–5 scale (0 = no galls, 1 = 1–2 galls, 2 = 3–10 galls, 3 = 11–30 galls, 4 = 31–100 galls, and 5 > 100 galls) (Taylor and Sasser 1978). Final population density of *M. incognita* in each pot was determined by processing each tomato root by the sodium hypochlorite method and extracting nematodes from 500-cm^3 soil of each pot by Coolen's method (1979) and then counting eggs and *J2*.

Data analysis

All experiments were repeated twice with separate controls for each experiment. Data from the two experimental runs of each experiment were pooled as no significant interaction of experiment × treatment resulted from a preliminary analysis of variance using experimental runs as factors (Finney 1978). Pooled data were arcsine-transformed, due to homogenization of error variances, and statistically analyzed using PlotIT 3.2 (Scientific Programming Enterprises, Haslett, MI). Data were subjected to one-way analysis of variance and treatment means compared using Fisher's least significant difference pairwise procedure at $p \leq 0.05$. The LC_{50} values of each EO and pure compounds on both nematode

Table 1 Amount (%± SD) of *M. didyma* and *M. fistulosa* EOs' components

No.	Compound	AI calc	AI tab	Identification method	%± SD	
					M. didyma	*M. fistulosa*
1	α-Thujene	929	931	GC, GC–MS	2.60 ± 0.11	4.01 ± 0.08
2	α-Pinene	931	939	GC, GC–MS	1.20 ± 0.04	1.21 ± 0.02
3	Camphene	952	953	GC, GC–MS	0.50 ± 0.03	–
4	Sabinene	974	976	GC, GC–MS	4.00 ± 0.17	0.28 ± 0.01
5	β-Pinene	977	980	GC, GC–MS	0.60 ± 0.02	0.30 ± 0.01
6	3-Octanone	981	986	GC–MS	2.92 ± 0.05	3.41 ± 0.15
7	Myrcene	992	991	GC, GC–MS	3.14 ± 0.06	4.42 ± 0.10
8	3-Octanol	998	993	GC–MS	0.75 ± 0.01	–
9	α-Phellandrene	1004	1005	GC, GC–MS	0.52 ± 0.01	0.63 ± 0.02
10	α-Terpinene	1020	1018	GC, GC–MS	3.62 ± 0.09	5.00 ± 0.12
11	*o*-Cymene	1030	1026	GC, GC–MS	13.42 ± 0.40	11.00 ± 0.21
12	Limonene	1035	1031	GC, GC–MS	2.40 ± 0.17	2.00 ± 0.03
13	Eucalyptol	1037	1033	GC, GC–MS	4.13 ± 0.08	1.00 ± 0.05
14	γ-Terpinene	1062	1062	GC, GC–MS	22.15 ± 0.54	25.20 ± 0.60
15	*cis*-Sabinene hydrate	1080	1068	GC–MS	0.30 ± 0.01	–
16	Terpinolene	1102	1088	GC, GC–MS	0.30 ± 0.01	–
17	Linalool	1117	1098	GC, GC–MS	8.01 ± 0.08	–
18	Borneol	1170	1165	GC–MS	0.42 ± 0.01	–
19	4-Terpineol	1179	1177	GC–MS	0.85 ± 0.02	0.80 ± 0.02
20	α-Terpineol	1190	1189	GC–MS	1.22 ± 0.03	0.53 ± 0.04
21	Thymol methyl ether	1248	1235	GC–MS	1.60 ± 0.02	4.70 ± 0.07
22	Carvacrol methyl ether	1249	1244	GC, GC–MS	2.80 ± 0.05	–
23	Isobornylacetate	1293	1285	GC, GC–MS	0.35 ± 0.00	–
24	Thymol	1303	1290	GC–MS	5.87 ± 0.37	8.36 ± 0.38
25	Carvacrol	1310	1298	GC–MS	13.80 ± 1.49	24.33 ± 1.47
26	β-Bourbonene	1403	1384	GC–MS	0.74 ± 0.01	–
27	β-Caryophyllene	1439	1418	GC–MS	1.20 ± 0.02	0.83 ± 0.01
28	β-Copaene	1441	1584	GC–MS	0.17 ± 0.13	0.72 ± 0.00
	Yield (%)				99.58	99.73

species were calculated by probit analysis of data from the in vitro assays.

Results

Chemical composition of essential oils

Qualitative and quantitative composition of the distilled EOs from *M. didyma* and *M. fistulosa* are reported in Table 1. Components identified by GC–FID and GC–MS averaged around 99% (99.58% in *M. didyma*; 98.73% in *M. fistulosa*) of total composition. A similar compositional profile was detected in the two samples of EOs, though additional constituents were found in the EO of *M. didyma* (Table 1). EOs from both species of *Monarda* were very rich in monoterpenes, with *o*-cymene, γ-terpinene, and carvacrol as the main constituents, amounting, respectively, to 13.42, 22.15, and 13.80% in *M. didyma* and to 11.00, 25.20, and 24.33% in *M. fistulosa*. Moreover, both EOs contained discrete amounts of thymol (5.87% and 8.36% in the EO of *M. didyma* and *M. fistulosa*, respectively). In addition, a significant content of linalool (8.01%) and low amounts of camphene (0.50%), 3-octanol (0.75%), *cis*-sabinene hydrate (0.30%), terpinolene (0.30%), borneol (0.42%), carvacrol methyl ether (2.80%), isobornylacetate (0.35%), and β-bourbonene (0.74%) were found only in the EO of *M. didyma*.

Nematode mortality bioassay

Mortality of both *M. incognita* and *P. vulnus* was nil or negligible either in sterilized water or in the Tween 20 solution (Table 2). EOs of *M. fistulosa* and *M. didyma* were strongly toxic to *M. incognita J2,* as LC50 values of both EOs ranged just 7.0 μg mL^{-1} after a 4-h exposure and were reduced to 1.0 μg mL^{-1} after the 24-h treatment (Table 2). The lowest tested concentration of both EOs caused more than 30% mortality of *M. incognita J2* after a 4-h treatment, whereas a 24-h immersion in the 12.5 μg mL^{-1} EOs' solution resulted in more than 80% mortality rates. Almost all the concentration × exposure time combinations of both *Monarda* EOs were more active on *M. incognita J2* than the nematicide Oxamyl solution.

Activity of the EOs from *M didyma* and *M. fistulosa* was largely lower on *P. vulnus* than on *M. incognita*, as LC50 values of 15.7 and 12.5 μg mL^{-1}, respectively, were

Table 2 Percentage mortality (mean ± SE) of infective *J2* of *M. incognita* and mixed stages of *P. vulnus* after a 4-, 8-, or 24-h exposure to a 3.12–100 μg mL^{-1} concentrations of EOs from *M. dydyma* and *M. fistulosa*

Treatment (μg mL^{-1})	Mortality (%)					
	4 h		8 h		24 h	
	M. incognita	*P. vulnus*	*M. incognita*	*P. vulnus*	*M. incognita*	*P. vulnus*
M. didyma						
3.12	32.1 ± 0.3	4.9 ± 0.4	37.3 ± 0.5	9.1 ± 0.4	65.5 ± 0.8	13.2 ± 0.8
6.25	44.5 ± 0.4	11.1 ± 0.6	45.7 ± 0.8	15.5 ± 0.5	73.2 ± 0.7	18.2 ± 0.5
12.5	72.6 ± 0.2	17.6 ± 0.2	69.6 ± 0.3	34.1 ± 0.6	82.7 ± 0.2	53.5 ± 0.7
25	71.9 ± 0.7	22.9 ± 0.3	76.7 ± 0.4	38.8 ± 0.1	87.1 ± 0.5	70.4 ± 0.8
50	73.6 ± 0.7	23.7 ± 0.1	79.8 ± 0.1	46.1 ± 0.4	91.6 ± 0.5	77.8 ± 0.3
100	82.5 ± 0.9	32.6 ± 0.2	87.4 ± 0.7	52.7 ± 0.8	95.2 ± 0.4	82.7 ± 0.6
LC50	7.0	≫[a]	6.1	63.8	1.0	15.7
M. fistulosa						
3.12	35.4 ± 0.2	5.1 ± 0.5	55.4 ± 0.3	7.2 ± 0.6	66.8 ± 0.9	12.3 ± 0.7
6.25	45.5 ± 0.7	9.7 ± 0.4	63.2 ± 06	13.5 ± 0.1	69.4 ± 0.6	26.2 ± 0.3
12.5	64.7 ± 0.7	13.2 ± 0.6	66.7 ± 0.4	18.8 ± 0.3	83.3 ± 0.3	67.6 ± 0.5
25	69.1 ± 0.9	19.1 ± 0.5	71.8 ± 0.9	24.2 ± 0.2	86.1 ± 0.6	75.1 ± 0.8
50	71.2 ± 0.9	19.6 ± 0.9	80.1 ± 0.3	30.1 ± 0.6	91.6 ± 0.1	77.8 ± 0.4
100	77.5 ± 0.9	29.9 ± 0.8	89.5 ± 0.9	46.7 ± 0.6	94.1 ± 0.2	84.1 ± 0.9
LC50	7.3	≫	2.2	≫	1.0	12.5
Oxamyl[b]	1.3 ± 0.1	33.3 ± 0.5	43.4 ± 0.2	61.1 ± 0.5	94.9 ± 0.7	64.9 ± 0.3
Tween 20[c]	0.1 ± 0.1	0.2 ± 0.2	1.7 ± 0.5	0.6 ± 0.2	4.2 ± 0.4	1.1 ± 0.4
Water	0.1 ± 0.1	0.1 ± 0.1	0.9 ± 0.5	0.4 ± 0.2	1.2 ± 0.4	0.7 ± 0.3
LSD 0.05	3.4	2.5	3.3	2.2	1.9	2.7

[a]LC50 values above the maximum tested concentration

[b]2.0 mL L^{-1} water

[c]0.3% water solution

calculated for the 24-h treatment of *P. vulnus* specimens and mortality rates peaked 83–84% values, respectively, only at the maximum exposure time × concentration combination (Table 2).

Both nematode species were highly sensitive to carvacrol, though the LC50 value of the 24-h treatment of *M. incognita* with carvacrol was less than half compared to that of *P. vulnus*, 14.2 versus 29.5 µg mL^{-1}, respectively (Table 3). Mortality rates of both nematodes ranged about 25% at the 4-h exposure to a 12.5 µg mL^{-1} carvacrol solution, but were consistently higher on *M. incognita* than on *P. vulnus* at the 24-h exposure to the maximum carvacrol concentration (82 vs. 70%, respectively).

Activity of γ-terpinene, *o*-cymene, and thymol was much lower than that of carvacrol on both nematode species, as also indicated by LC50 values largely above the maximum tested concentration (Table 3). A 24-h exposure to a 100 µg mL^{-1} solution of γ-terpinene caused ranged

Table 3 Percentage mortality (mean ± SE) of infective *J2* of *M. incognita* and mixed stages of *P. vulnus* after a 4-, 8-, or 24-h exposure to 3.12–100 µg mL^{-1} concentrations of carvacrol, γ-terpinene, *o*-cymene, and thymol

Treatment (µg mL^{-1})	4 h		8 h		24 h	
	M. incognita	*P. vulnus*	*M. incognita*	*P. vulnus*	*M. incognita*	*P. vulnus*
Carvacrol						
3.12	12.9 ± 0.3	15.7 ± 0.3	19.3 ± 0.2	21.7 ± 0.5	31.4 ± 0.2	28.8 ± 0.2
6.25	19.6 ± 0.3	17.5 ± 0.4	24.0 ± 0.3	23.9 ± 0.2	36.1 ± 0.3	31.8 ± 0.1
12.5	25.8 ± 0.3	25.3 ± 0.4	31.1 ± 0.3	30.3 ± 0.1	42.7 ± 0.7	39.1 ± 0.3
25	29.8 ± 0.3	30.5 ± 0.1	36.8 ± 0.4	35.2 ± 0.4	47.2 ± 0.2	43.8 ± 0.3
50	33.1 ± 0.2	36.7 ± 0.2	43.1 ± 0.1	41.3 ± 0.2	79.8 ± 0.2	51.7 ± 0.1
100	37.7 ± 0.4	45.2 ± 0.3	47.4 ± 0.3	61.1 ± 0.4	82.6 ± 0.3	70.7 ± 0.2
LC50	»[a]	»	»	68.2	14.2	29.5
γ-Terpinene						
3.12	4.1 ± 0.4	3.3 ± 0.3	7.6 ± 0.3	7.6 ± 0.3	8.2 ± 0.4	13.7 ± 0.4
6.25	5.1 ± 0.3	5.1 ± 0.3	10.2 ± 0.3	10.2 ± 0.2	13.4 ± 0.2	19.6 ± 0.3
12.5	6.8 ± 0.3	5.6 ± 0.3	11.4 ± 0.5	14.5 ± 0.5	19.8 ± 0.3	25.2 ± 0.3
25	9.1 ± 0.3	7.7 ± 0.4	13.8 ± 0.2	19.4 ± 0.4	27.1 ± 0.5	31.5 ± 0.2
50	10.9 ± 0.4	11.1 ± 0.4	17.3 ± 0.3	23.4 ± 0.3	31.3 ± 0.8	34.6 ± 0.1
100	14.2 ± 0.4	16.4 ± 0.5	20.5 ± 0.5	27.4 ± 0.3	51.8 ± 0.3	45.3 ± 0.5
LC50	»	»	»	»	118.3	»
o-Cymene						
3.12	0.8 ± 0.1	6.6 ± 0.4	5.2 ± 0.5	9.5 ± 0.3	13.1 ± 0.3	22.9 ± 0.4
6.25	1.7 ± 0.3	7.6 ± 0.3	6.3 ± 0.1	12.8 ± 0.4	18.9 ± 0.4	27.5 ± 0.5
12.5	3.3 ± 0.3	11.8 ± 0.4	9.1 ± 0.2	15.6 ± 0.4	24.3 ± 0.4	33.7 ± 0.4
25	4.4 ± 0.2	13.4 ± 0.3	11.7 ± 0.3	19.4 ± 0.2	30.1 ± 0.3	37.1 ± 0.4
50	4.9 ± 0.1	16.6 ± 0.2	15.5 ± 0.4	22.6 ± 0.4	38.3 ± 0.4	42.5 ± 0.4
100	7.8 ± 0.3	19.6 ± 0.4	18.2 ± 0.3	27.5 ± 0.2	43.9 ± 0.2	54.9 ± 0.2
LC50	»	»	»	»	»	82.9
Thymol						
3.12	1.5 ± 0.3	5.6 ± 0.3	7.2 ± 0.3	9.3 ± 0.3	21.1 ± 0.2	14.2 ± 0.4
6.25	5.7 ± 0.3	6.8 ± 0.4	8.8 ± 0.3	11.1 ± 0.3	29.3 ± 0.2	19.8 ± 0.3
12.5	6.6 ± 0.3	9.2 ± 0.3	12.2 ± 0.3	13.1 ± 0.4	32.8 ± 0.3	22.7 ± 0.4
25	10.1 ± 0.3	10.6 ± 0.2	15.9 ± 0.3	17.3 ± 0.2	37.7 ± 0.2	26.3 ± 0.1
50	11.8 ± 0.5	15.8 ± 0.3	19.7 ± 0.5	19.5 ± 0.2	40.3 ± 0.1	28.8 ± 0.2
100	18.1 ± 0.4	17.4 ± 0.2	23.1 ± 0.3	21.1 ± 0.2	43.5 ± 0.3	34.4 ± 0.3
LC50	»	»	»	»	»	»
Oxamyl[b]	2.2 ± 0.1	31.0 ± 0.3	43.0 ± 0.8	61.5 ± 0.3	92.8 ± 0.3	68.2 ± 0.2
Tween 20[c] 0.03%	0.5 ± 0.3	0.2 ± 0.2	1.8 ± 0.1	1.4 ± 0.4	1.8 ± 0.3	1.4 ± 0.3
Control	0.2 ± 0.2	0.2 ± 0.2	0.7 ± 0.5	0.3 ± 0.2	0.7 ± 0.1	0.3 ± 0.1
LSD 0.05	0.9	0.9	1.1	0.9	1.1	1.0

[a]LC50 values above the maximum tested concentration

[b]2.0 mL L^{-1} water

[c]0.3% water solution

51.8–54.9% peak mortality rates on *M. incognita* and *P. vulnus*, respectively, whereas the same treatment with *o*-cymene solution peaked 43.9 and 45.3% mortality, respectively. Thymol was less active than the other three tested compounds, as a maximum of 43.5 and 34% mortality was recorded for the 24-h treatment of *M. incognita J2* and *P. vulnus* specimens, respectively, with a 100 µg mL^{-1} thymol solution.

Binary combinations of the three main compounds of *Monarda* EOs, carvacrol, γ-terpinene, and *o*-cymene, were poorly active on *M. incognita* (Table 4). All the combinations of the three terpenes resulted in higher mortality rates of *P. vulnus* compared to the same treatment with the single compounds, though the strongest effect resulted for the 1:2 *o*-cymene: carvacrol mixture.

Egg hatching bioassay

Percentage hatch of *M. incognita* eggs treated with 500 and 1000 µg mL^{-1} solutions of *M. didyma* and *M. fistulosa* EOs was always significantly lower than water and Tween 20 controls and even than the 24- and 48-h treatment with the Oxamyl solution (Fig. 1a, b). The eggs treated with the *M. didyma* EO showed a significantly lower hatch percent compared to those exposed to the EO of *M. fistulosa* but at the maximum time × concentration combination. Differences between the two tested concentrations were significantly different only at the 24-h treatment with the *M. didyma* EO.

Both concentrations of carvacrol and thymol resulted in a strong inhibition of *M. incognita* egg hatch, reduced to only 4.1 and 15.3%, respectively, by the 48-h treatment with the 1000 µg mL^{-1} solution of these compounds (Fig. 1c, d). However, most of the treatments with carvacrol resulted in a significantly lower hatch percent compared to thymol. Both concentrations of γ-terpinene and *o*-cymene significantly reduced the hatch of *M. incognita* eggs only at the 48-h treatment.

Experiment in soil

Treatments with both EOs of *M. didyma* and *M. fistulosa* resulted in a significant reduction of *M. incognita* eggs and *J2* either on plant roots and in soil or, almost generally, also of gall formation, on tomato roots (Table 5). The suppressive effect of 500 and 1000 µg kg^{-1} soil rates of both EOs was statistically not different from that of the treatment with Oxamyl. Aggregate effects on nematode density on plant roots and in soil were significantly higher for the EO *M. didyma* and for the rates ≥ 250 µg mL^{-1}.

Treatments with both EOs always resulted also in a significantly heavier biomass of aerial parts and roots of tomato plants, compared either to the untreated control and Oxamyl or Tween 20 treatments (Table 5). The aggregate growth effect was significantly larger at the two lowest EO rates and, limited to root biomass, for the EO of *M. didyma*.

Table 4 Percentage mortality (mean ± SE) of infective *J2* of *Meloidogyne incognita* and mixed stages of *P. vulnus* after a 24-h exposure to 6.25–25 µg mL^{-1} concentrations of selected combinations of carvacrol, γ-terpinene, and *o*-cymene

Combination	Ratio	Rate (µg mL^{-1})	Mortality (%)	
			M. incognita	*P. vulnus*
o-Cymene: carvacrol	2:1	6.25	23.3 ± 0.4	40.8 ± 0.3
	2:1	12.5	26.5 ± 04	45.7 ± 0.4
	2:1	25	29.6 ± 01	47.1 ± 0.1
o-Cymene: carvacrol	1:1	6.25	16.5 ± 0.4	37.4 ± 0.3
	1:1	12.5	21.1 ± 0.4	43.8 ± 0.4
	1:1	25	27.1 ± 0.4	47.1 ± 0.1
o-Cymene: carvacrol	1:2	6.25	21.0 ± 0.2	46.1 ± 0.2
	1:2	12.5	27.1 ± 0.2	52.0 ± 0.1
	1:2	25	31.5 ± 0.2	60.7 ± 0.4
γ-Terpinene: carvacrol	2:1	6.25	19.3 ± 0.9	34.0 ± 0.3
	2:1	12.5	22.5 ± 0.3	36.2 ± 0.3
	2:1	25	32.7 ± 0.3	44.5 ± 0.3
γ-Terpinene: carvacrol	1:1	6.25	14.5 ± 0.4	29.6 ± 0.4
	1:1	12.5	20.1 ± 0.4	34.9 ± 0.1
	1:1	25	29.3 ± 0.4	40.3 ± 0.2
γ-Terpinene: carvacrol	1:2	6.25	27.1 ± 0.1	37.8 ± 0.4
	1:2	12.5	30.4 ± 0.3	51.7 ± 0.4
	1:2	25	36.1 ± 0.4	57.4 ± 0.4
γ-Terpinene: *o*-cymene	2:1	6.25	15.1 ± 0.3	31.1 ± 0.3
	2:1	12.5	17.8 ± 0.2	34.5 ± 0.4
	2:1	25	20.3 ± 0.2	42.2 ± 0.3
γ-Terpinene: *o*-cymene	1:1	6.25	13.2 ± 0.3	38.7 ± 0.4
	1:1	12.5	14.5 ± 0.3	45.2 ± 0.2
	1:1	25	19.2 ± 0.3	54.3 ± 0.4
γ-Terpinene: *o*-cymene	1:2	6.25	10.6 ± 0.2	43.7 ± 0.4
	1:2	12.5	13.7 ± 0.2	50.7 ± 0.4
	1:2	25	17.7 ± 0.4	54.6 ± 0.3
Oxamyl[a]	–		92.8 ± 0.3	68.2 ± 0.3
Tween 20[b]	–		1.8 ± 0.3	1.2 ± 0.3
Water	–	–	0.7 ± 0.1	0.1 ± 0.1
LSD 0.05		–	1.1	0.9

[a]2.0 mL L^{-1} water

[b]0.3% water solution

Discussion

Biological activities proved for *Monarda* species have been related to the terpenoid composition of their EOs, though several chemotypes have been described. In particular, four different chemotypes are reported for *M. didyma* and *M. fistulosa*, with EOs containing either linalool, geraniol,

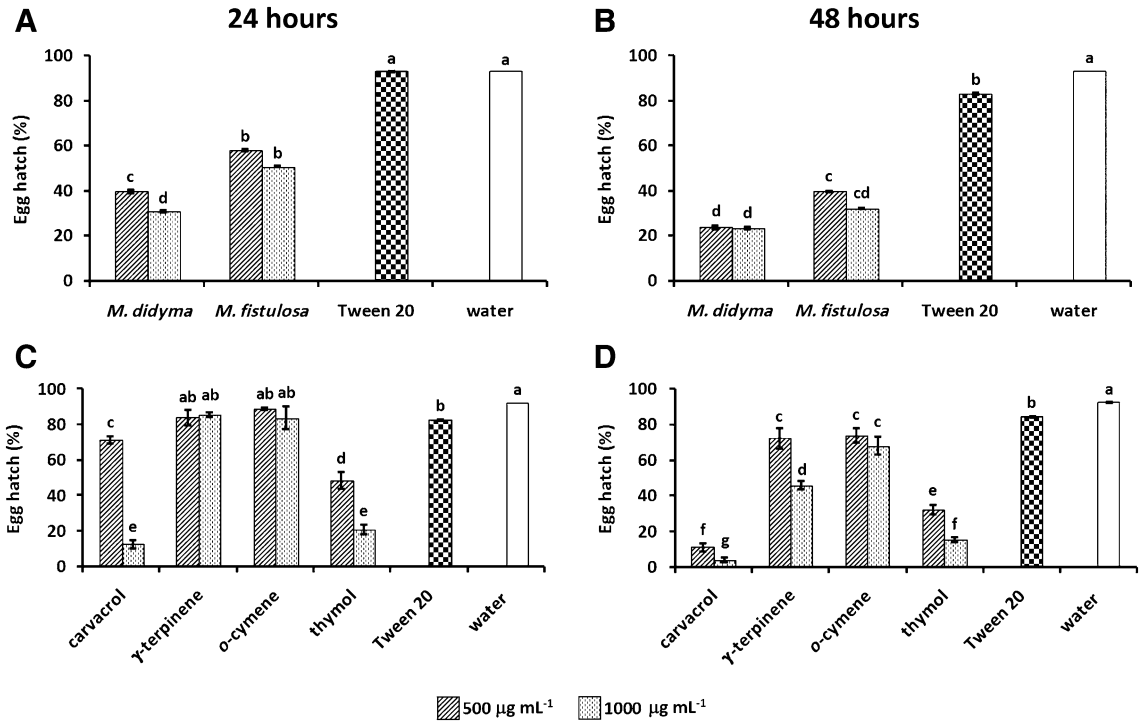

Fig. 1 Effect on percentage egg hatch of a 24- or 48-h exposure of egg masses of *M. incognita* to 500 and 1000 µg mL^{-1} solutions of the EOs of *M. dydima* and *M. fistulosa* (**a**, **b**) or of their main components, carvacrol, γ-terpinene, *o*-cymene, and thymol (**c**, **d**)

carvacrol, or thymol as major terpenes (Gwinn et al. 2010; Mattarelli et al. 2017). In agreement, the EOs from *M. didyma* and *M. fistulosa* used in this study are characterized by a high content of carvacrol and *o*-cymene, a discrete amount of thymol, whereas the dominant component is represented by γ-terpinene, as revealing an unusual compositional profile for both species.

Among the various biological properties of *M. didyma* and *M. fistulosa* EOs, a larger attention should be deserved to their biocidal activity, as documented until now only on fungal phytopathogens and insects. Both contact and volatile phases of *M. didyma* and *M. fistulosa* EOs were found to inhibit spore germination and mycelial growth of gray mold agent *B. cinerea* (Adebayo et al. 2013). The inhibitory effects of *Monarda* EOs were documented also on the white mold agent *Sclerotinia sclerotiorum* (Lib.) de Bary and the soilborne pathogen *Rhizoctonia solani* K. (Fraternale et al. 2006; Gwinn et al. 2006). Soil amendments with dry leaf material of *M. didyma* were reported for a suppressive activity on plant fungal pathogens, in planting media (Gwinn et al. 2006, 2007), as well as positively tested in greenhouse against fungal agents of peanut and tomato damping-off or crown rot, including *R. solani* and *Aspergillus niger* van Thieghem (Gwinn et al. 2010; Ruark and Shew 2010). Data on the insecticidal activity of *Monarda* EOs are limited only to the report of a repellent activity of *M. fistulosa* EO on the yellow fever mosquito *Aedes aegypti* L. (Tabanca et al. 2013).

To the best of our knowledge, this is the first report of the nematicidal activity of the EOs from *Monarda* plants, though differentiated by nematode species. Data presented in this work indicated both *M. didyma* and *M. fistulosa* EOs as highly active on *M. incognita J2* after short exposures to low concentrations, whereas the infective stages of *P. vulnus* were consistently affected only at the highest tested concentrations.

A nematicidal activity has been widely reported for the EOs from several plant species of various botanical and geographical origin (Andrés et al. 2013; Laquale et al. 2015; Avato et al. 2017). Nematicidal effects of terpenes commonly found as major components of EOs have been also largely investigated (Oka 2001; Echeverrigaray et al. 2010; Avato et al. 2017). Results of this study showed that carvacrol was more toxic than thymol, γ-terpinene, and *o*-cymene to infective stages of *M. incognita* and *P. vulnus*. Mortality rates of the two nematodes were comparable at carvacrol concentrations above 12.5 µg mL^{-1}, whereas the specimens of *P. vulnus* were more sensitive than *M. incognita J2* at the lowest concentrations. In addition, carvacrol resulted also in the strongest inhibitory effect on *M. incognita* egg hatch, followed, at a less extent, by thymol. Compared to carvacrol, thymol, γ-terpinene, and *o*-cymene showed a much lower toxicity to the infective stages of both phytonematodes and

Table 5 Effect of soil treatments with the EOs from *M. didyma* and *M. fistulosa* at rates of 62.5–1000 µg kg^{-1} soil on the infestation of the root-knot nematode *Meloidogyne incognita* on tomato cv. Roma and on plant growth (mean ± SE)

Treatment	Nematode population (eggs and *J2*)		Root gall index	Plant fresh weight (g)	
	g^{-1} roots	mL^{-1} soil		Aerial parts	Roots
M. didyma					
62.5	3047 ± 53	3.5 ± 0.1	3.4 ± 0.5	35.3 ± 0.7	11.1 ± 0.5
125	2849 ± 106	3.3 ± 0.1	3.2 ± 0.5	38.1 ± 0.8	10.9 ± 0.5
250	2393 ± 55	2.8 ± 0.1	3.0 ± 0.3	30.6 ± 0.5	9.4 ± 0.3
500	1792 ± 58	2.5 ± 0.1	2.4 ± 0.4	26.8 ± 0.4	7.5 ± 0.2
1000	1272 ± 36	2.3 ± 0.1	2.4 ± 0.4	26.6 ± 0.6	7.4 ± 0.1
M. fistulosa					
62.5	3891 ± 65	4.1 ± 0.1	3.8 ± 0.4	34.5 ± 0.6	8.7 ± 0.1
125	3667 ± 61	3.7 ± 0.1	3.6 ± 0.2	35.5 ± 0.7	8.6 ± 0.2
250	2614 ± 93	3.4 ± 0.2	3.2 ± 0.5	31.8 ± 1.5	9.0 ± 0.2
500	2326 ± 39	3.3 ± 0.1	3.0 ± 0.3	30.7 ± 0.4	8.4 ± 0.2
1000	2055 ± 40	2.9 ± 0.1	2.6 ± 0.4	25.8 ± 0.2	6.4 ± 0.1
Oxamyl[a]	2013 ± 36	2.4 ± 0.1	2.6 ± 0.4	22.9 ± 0.9	6.4 ± 0.1
Tween 20[b]	4129 ± 19	5.8 ± 1.1	4.4 ± 0.2	16.6 ± 0.1	3.7 ± 0.1
Non-treated	4152 ± 16	5.6 ± 0.1	4.6 ± 0.4	18.5 ± 0.6	3.9 ± 0.1
Non-infested	–	–	–	23.0 ± 0.9	9.2 ± 0.1
LSD	164	0.3	1.1	2.0	0.7
Two-way ANOVA: EOs					
M. dydima	2271 ± 137	3.6 ± 0.1	2.9 ± 0.2	31.5 ± 1.0	9.3 ± 0.4
M. fistulosa	2910 ± 152	4.4 ± 0.1	3.2 ± 0.2	31.7 ± 0.8	8.2 ± 0.2
Student's *t*[c]	*	*	–	–	*
Two-way ANOVA: EOs' rates					
62.5	3469 ± 146	3.8 ± 0.1	3.6 ± 0.3	34.9 ± 0.5	9.9 ± 0.5
125	3258 ± 148	3.5 ± 0.1	3.4 ± 0.3	36.8 ± 0.7	9.8 ± 0.5
250	2504 ± 63	3.1 ± 0.2	3.1 ± 0.3	31.2 ± 0.8	9.2 ± 0.2
500	2059 ± 95	2.9 ± 0.1	2.7 ± 0.3	28.8 ± 0.7	8.0 ± 0.2
1000	1664 ± 133	2.6 ± 0.1	2.5 ± 0.3	26.2 ± 0.3	6.9 ± 0.2
LSD	346	0.4	0.8	1.7	0.9

[a] $mL\ kg^{-1}$ soil

[b] 0.3% water solution

[c] significant at $P = 0.05$

to the root-knot nematode eggs. Carvacrol is a common component of several EOs from aromatic plants, known for several biological properties including also nematicidal activity (Can Baser 2008; Zotti et al. 2013). Carvacrol was documented for a high in vitro toxicity to *J2* and eggs of *M. javanica* and *M. incognita* (Oka et al. 2000; Ibrahim et al. 2006), and soil treatments with this compound were found to strongly reduce or even completely suppress the infestation of *M. javanica* on tomato (Oka et al. 2000). Thymol was also documented for a high toxicity to root-knot nematode *J2* (Ntalli et al. 2010; Santana et al. 2014), as recently confirmed by Avato et al. (2017), that recorded a 57% mortality rate after a 48-h exposure of *M. incognita J2* to a 15 µg mL^{-1} thymol solution. The same thymol solution was much less active on *P. vulnus*, as causing 27% mortality only after a 96-h treatment. To the best of our knowledge, there are no previous reports on the activity of γ-terpinene and *o*-cymene on phytoparasitic nematodes. A different sensitivity of nematode species tested in our work to phytochemicals has also been reported in previous studies (Al-Banna et al. 2003; D'Addabbo et al. 2013; Ntalli et al. 2013) and is suggested to be related to a different anatomy and feeding behavior (Yeats et al. 1993; Davies and Curtis 2011, Avato et al. 2017). Thus, for example, EO from *C. sinensis*, containing 96% of limonene, was highly active against *P. vulnus*, but only poorly toxic to *M. incognita* (Avato et al. 2017).

None of the four main constituents of *M. didyma* and *M. fistulosa* EOs were as toxic to *M. incognita* and *P. vulnus* as the whole EOs. Moreover, the two EOs were generally much more toxic than single carvacrol to *M. incognita*, whereas 4- and 8-h treatments with carvacrol caused a higher mortality of *P. vulnus* than the whole EOs. This may suggest

the occurrence of synergistic or antagonistic effects of EOs' main compounds on *M. incognita* and *P. vulnus*, respectively, as well as the contribution to the nematicidal activity of further minor compounds not tested in this study. Consistently, binary mixtures of carvacrol with the other two main EOs' components (γ-terpinene and *o*-cymene) were always less toxic to *M. incognita J2* than single carvacrol, though an increase of nematotoxic activity was observed by increasing the carvacrol amount (*o*-cymene: carvacrol and γ-terpinene: carvacrol, 1:2). In contrast, the activity of paired combinations of the three main terpenes on *P. vulnus* was higher compared to that of single compounds though lower toxicity of the whole EOs.

Mechanisms of the activity of EOs and their constituents to phytoparasite nematodes are still unclear, though different hypotheses are suggested in literature including a disruption and change of permeability of nematode cell membranes or the inhibition of AChE activity (Andrés et al. 2013). However, previous investigations have indicated the nematicidal activity of plant EOs as related to the presence of selected terpenoids in their chemical structures (Oka 2001; Echeverrigaray et al. 2010; Laquale et al. 2015). Therefore, according to the results obtained with the two *Monarda* EOs and their main pure compounds, it may be suggested that main terpenes contained in the EOs of *M. didyma* and *M. fistulosa* highly contribute to their nematotoxic effect against *M. incognita* and *P. vulnus.* However, the almost similar chemical composition of the two tested EOs suggests that their different behavior to the two nematode species could not be merely attributed to EOs' chemical features, as a variability intrinsic to the nematode species should be also considered, maybe related to the above cited different anatomy and feeding habitus.

Conclusion

The results of this study indicated the EOs from *M. didyma* and *M. fistulosa* as highly toxic to phytoparasitic nematodes and particularly to root-knot nematodes, the most harmful to most economically relevant crops. Therefore, the *Monarda* EOs may have a large potential for the development of new nematicidal products to include in sustainable strategies for nematode management, also considering the current scarcity of effective synthetic nematicides.

The positive effects showed by soil drench treatments with the two EOs also demonstrated the technical feasibility of soil application of these potential nematicide products by drip irrigation, the most frequently applied irrigation technique in intensive crop systems.

Chemical and physical features of EOs, mainly their high volatility and quick dispersion and degradation, impose also the search for suitable technical formulations of EO-based nematicides, such as their micro- or nano-encapsulation (Martín et al. 2010), which can provide a controlled release and a slow degradation of EOs' active components and, therefore, can prolong and enhance the nematicidal efficacy of soil treatments with EOs.

Finally, it should be considered that the content of active compounds responsible for the EOs' nematicidal activity is highly affected by climatic and agronomical factors, as well as by the extraction technique. Therefore, standardized techniques of plant cultivation and EO extraction should also be taken into consideration in order to formulate commercial products with a standardized composition and nematicidal efficacy.

Author contributions

SL and TD conceived and designed the research and carried out the experiments on nematodes. PA supervised the chemical analyses and drafted the manuscript. MPA carried out the chemical analyses. MGB provided plant materials and extracted essential oils. TD analyzed the data and edited the manuscript. All the authors read and approved the manuscript.

Compliance with 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 or animals performed by any of the authors.

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