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Nematicidal activity of essential oils and organic amendments from Asteraceae against root-knot nematodes

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The essential oil of *Chrysanthemum coronarium* flowerheads showed strong nematicidal activity *in vitro* and in growth-chamber experiments. Essential oil concentrations of 2, 4, 8 and 16 μL mL^{-1}, significantly reduced hatch, J_2 survival (determined by final value and area under curves of cumulative percentage hatch or mortality) and reproduction rate of *Meloidogyne artiellia in vitro*, with the lowest values occurring at 16 μL mL^{-1}. In pot trials with chickpea cv. PV 61, essential oil concentrations of 10–40 μL per 500 cm^3 soil, applied on sterile cotton pellets, also significantly reduced the nematode's reproduction rate. The biological processes of mortality and hatching/reproduction were adequately described by the monomolecular and expanded negative exponential models, respectively. Effectiveness of soil amendment with either flowers, leaves, roots or seeds of *C. coronarium*, and flowers from several species of Asteraceae (*Chrysanthemum segetum*, *Calendula maritima*, *Calendula officinalis* and *Calendula suffruticosa*) at 5 g per 500 cm^3 soil was tested for suppression of *M. artiellia* and growth of chickpea cv. PV 61 under growth-chamber conditions. In these tests, flowers of all five Asteraceae species and various parts of *C. coronarium* significantly reduced reproduction rates of *M. artiellia*, by 83·0–95·9%, with the minimum rates occurring in infected chickpea plants amended with flowers of *C. officinalis* and *C. suffruticosa*. The *in vitro* and *in planta* results suggest that the essential oil of *C. coronarium* and organic amendments from Asteraceae species may serve as nematicides.

Keywords: *Calendula maritima*, *Calendula officinalis*, *Calendula suffruticosa*, *Chrysanthemum coronarium*, *Chrysanthemum segetum*, *Meloidogyne artiellia*, nematicide

Introduction

Plant-parasitic nematodes are an economically important group of soilborne pathogens that may be controlled by cultural practices, chemical nematicides and the use of resistant cultivars. However, nematicides do not provide long-term suppression of nematodes, and environmental and human health concerns are resulting in increased restrictions on their use. Some safe procedures for nematode control have been developed based on biological control agents and organic amendments; however, there is still a need for alternative, environmentally friendly measures or compounds for effective nematode control to be developed (Noling & Becker, 1994). One way of searching for such nematicidal compounds is to screen naturally occurring compounds in plants.

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Plants are an important source of naturally occurring pesticides. Many compounds with nematicidal activity have been found in plants, including alkaloids, diterpenes, fatty acids, glucosinolates, isothiocyanates, phenols, polyacetylenes, sesquiterpenes and thienyls (Gommers, 1981; Chitwood, 2002). Many compounds with nematicidal activity have been isolated from species in the family Asteraceae (Gommers, 1981; Chitwood, 2002). Essential oils of some plants and/or their components have been tested for nematicidal activity *in vitro* and in soil (Chatterjee *et al*., 1982; Soler-Serratosa *et al*., 1996; Oka *et al*., 2000). Recently, the antifungal and insecticidal activity of the essential oil of *Chrysanthemum coronarium* flowerheads has been reported (Pérez & Pascual-Villalobos, 1999; Alvarez-Castellanos *et al*., 2001). However, there is no information about the activity of these compounds against nematodes.

The cereal root-knot nematode *Meloidogyne artiellia* has been found to be the causal agent of severe yield losses of wheat and barley in Greece (Sikora, 1987), and especially of chickpea in the Mediterranean Basin (Tobar

Jiménez, 1973; Greco, 1984; Di Vito & Greco, 1988), where 10–12% of stunted chickpeas were infected with the nematode. In addition, investigations on the host range of this root-knot nematode indicated that, with the exception of oat, corn and lentil, *M. artiellia* reproduces well on cereals, cruciferous plants and legumes (Di Vito *et al*., 1985). These crops are grown extensively in the Mediterranean Basin and severe damage can be expected in infested fields. The objectives of this work were: (i) to evaluate the effect of the essential oil of *C. coronarium* flowerheads on hatching of *M. artiellia* eggs and viability of second-stage juveniles; and (ii) to determine the nematicidal activity of organic amendments from several plant parts and species of the family Asteraceae against *M. artiellia*.

Materials and methods

Plant material and essential oils

The plants and plant parts used as organic amendments and for the extraction of essential oils are listed in Table 1. Plant material was obtained from plants grown in an experimental plot (Torreblanca Experimental Station, Campo de Cartagena, Murcia, Spain). Plants were sown in autumn (26 October 1995) and harvested in April of the following year. Voucher specimens (no. 207) were deposited in the Centro de Investigación y Desarrollo Agroalimentario Herbarium (CIDAHERB, Murcia, Spain). After harvest, plant-part samples were frozen at –40°C for 24 h and lyophilized. Lyophilized materials were crushed in a grinder to obtain particles *c*. 1 mm long.

Essential oil of *C. coronarium* flowerheads was obtained by the hydrodistillation method using a Clevenger-type apparatus. Before distillation, the flowerheads were lyophilized. The yield of oil was about 0·1% (v/w) and it had a blue colour and a disagreeable smell. The chemical composition of essential oils was determined by GC/MS (Alvarez-Castellanos *et al*., 2001). The main compounds identified were camphor (29·2%), α- and β-pinene (14·8 and 9·5%, respectively) and lyratil acetate (9·8%) (Alvarez-Castellanos *et al*., 2001).

Nematode inoculum

The nematode population used in the study was obtained from roots of chickpeas (cv. Ghab 1) collected in commercial fields at Monopoli, Italy. To establish and maintain cultures of *M. artiellia*, a single egg mass from a single gall containing a single female was removed from a root, surface-sterilized with 1% NaOCl for 4 min, and rinsed through four series of sterilized water. To increase nematode populations, a single egg mass was inoculated into a pot containing a 2–3-week-old chickpea plant (cv. UC 27) in sterilized soil and kept at 20–22°C for 3–4 months. More cultures were then raised in the same conditions to increase the nematode inoculum to the amount required for experiments. Egg inocula were prepared according to the NaOCl procedure (Hussey & Barker, 1973). Washed *M. artiellia*-infected chickpea roots were cut into small segments (1–2 cm long) and agitated for 3 min in 1% NaOCl. The suspension was passed through 75 and 5 μm sieves. The eggs and second-stage juveniles (J_2) caught on the 5 μm sieve were washed several times with water, resuspended, and their concentration determined by dilution counts. For the hatching test, the J_2s remaining in the egg suspension were removed by hand under a stereomicroscope. Nematodes [10 000 (eggs + J_2s) per plant] were added in 10 mL suspension around the radicle of pregerminated seeds at sowing. Control plants were treated similarly with sterile distilled water.

In vitro experiments

Influence of essential oils on hatch

Hatching was monitored in chambers composed of 20 mm diameter microsieves (75 μm aperture) enclosed in Petri dishes containing sterile deionized distilled water (SDDW) covering the egg masses. Ten mature, uniformly sized egg masses, with mean viable egg contents of 225, were placed in each of six replicate hatching chambers for each essential oil concentration. Essential oil was added to the Petri dishes to obtain a final concentration. Essential oil solutions (10% ethanol, v/v) of *C. coronarium* were diluted with SDDW containing 0·3% Tween 20 (v/v) was added to the Petri dishes at final concentrations of 2, 4, 8 and 16 μL mL^{-1}. The SDDW, with concentrations of ethanol and Tween 20 equivalent to those in treatments with essential oil, was used as the control. Petri dishes were maintained at 20°C (±1°C) in the dark for 4 weeks. The numbers of J_2s that emerged were recorded at 2–3-day intervals for 4 weeks. After 4 weeks, egg masses from each hatching chamber were separated to estimate the number of unhatched eggs, and the number of hatched J_2s

Table 1 Plant species used for organic amendments and essential-oil extraction

Botanical name	Plant part	Seed origin
Chrysanthemum coronarium	Flowers	USDA-PI586600, Korea
Chrysanthemum coronarium	Leaves	USDA-PI586600, Korea
Chrysanthemum coronarium	Roots	USDA-PI586600, Korea
Chrysanthemum coronarium	Seeds	USDA-PI586600, Korea
Chrysanthemum segetum	Flowers	RBG, Kew-29795, UK
Calendula officinalis	Flowers	University of Göttingen, Germany
Calendula maritima	Flowers	ETSIA, Madrid, Spain
Calendula suffruticosa ssp. *algarbiensis*	Flowers	ETSIA, Madrid, Spain

Table 2 Influence of organic amendments from several Asteraceae on the growth of chickpea cv. PV 61 and reproduction of *Meloidogyne artiellia*[a]

Treatment or plant species	Plant part	Nematode inoculum (eggs + J_2 per cm^3 soil)	Plant growth (g)		R_f[b] (P_f/P_i)
			Shoot DW	Root FW	
Uninoculated control	–	0	1·18 a	12·76 a	0
Inoculated control	–	20	1·18 a	13·45 a	54·5 a
Chrysanthemum coronarium	Flowers	20	1·39 a	13·40 a	3·8 b
Chrysanthemum coronarium	Leaves	20	1·43 a	15·06 a	9·3 bc
Chrysanthemum coronarium	Roots	20	1·35 a	12·31 a	8·0 bcd
Chrysanthemum coronarium	Seeds	20	1·53 a	11·93 a	5·1 bcd
Chrysanthemum segetum	Flowers	20	1·25 a	12·80 a	3·9 cd
Calendula officinalis	Flowers	20	1·30 a	10·88 a	2·8 cd
Calendula maritima	Flowers	20	1·04 a	10·93 a	5·3 d
Calendula suffruticosa ssp. *algarbiensis*	Flowers	20	1·16 a	8·15 a	2·2 d

[a]Data are the averages of two experiments with five replicate plants per treatment combination in each experiment. Means followed by the same letter do not differ significantly ($P > 0·05$) according to Fisher's protected LSD test.

[b]R_f (nematode reproduction rate) = final nematode density per plant/initial nematode population density per plant. Data were transformed to log ($X + 1$) for analysis.

was expressed as a cumulative percentage of viable J_2s. Experimental treatments were completely randomized in the incubator. The experiment was repeated once.

Nematicidal activity of essential oils on J_2*s*

The nematicidal activity of the essential oil of *C. coronarium* was evaluated against J_2s of *M. artiellia*. Mature egg masses of the nematode were placed in SDDW for 72 h in hatch chambers, as previously described. The J_2s emerging were collected every 24 h, but those collected in the first 24 h were discarded. A 5 mL suspension of about 100 J_2s was poured into six replicate 5 cm Petri dishes for each essential oil concentration. Essential oil solution (10% ethanol, v/v) was diluted with water containing 0·3% Tween 20 (v/v) and added to the Petri dishes to obtain final concentrations of 1, 2, 4, 8 and 16 μL mL^{-1}. The SDDW, with equivalent concentrations of ethanol and Tween 20 to those in the essential oil treatments, was used as a control. Petri dishes were maintained at 20°C (±1°C) in the dark for 10 days. The J_2s were observed under a stereoscopic microscope after 1, 2, 3, 4, 6, 8 and 10 days, and percentage mortality was calculated from the number of J_2s that did not move even after pricking the tail. The experiment was repeated once.

In planta experiments

Effect of organic amendments

This experiment was carried out under a controlled environment in a growth chamber adjusted to 22 ± 1°C, 60–90% relative humidity and a 14 h photoperiod of fluorescent light at 360·5 ± 24·7 μE m^{-2} s^{-1}. These environmental conditions are favourable for development and reproduction of *M. artiellia* (Di Vito & Greco, 1988). Germinated chickpea seeds (cv. PV 61) were sown in clay pots (one per pot) containing 0·5 L of an autoclaved soil potting mixture (sand : clay loam, 2 : 1, v/v) which, for the experimental treatments, was amended with 5 g lyophilized and homogenized plant material (representing ≈1% of the soil substrate, v/v). Plant species and plant parts used as soil amendments are listed in Table 1. For the nematode inoculation, 10 mL sterile distilled water with nematode inoculum [10 000 (eggs + J_2s)] were added to soil at sowing. There were 10 treatment combinations (Table 2) arranged in a completely randomized design and replicated six times, each replicate consisting of a single potted plant. Plants were watered daily with 100 mL tap water and fertilized weekly with 100 mL 0·1%, 20-5-32 + micronutrients hydro-sol fertilizer (Haifa Chemicals Ltd, Haifa, Israel).

The experiment was terminated 62 days after inoculation, when plants were at the full-bloom to early podding stage. The shoot of each plant was cut off at soil level and the roots washed free of soil. The following variables were assessed: dry shoot and fresh root weight; number of nematodes in roots and soil; and nematode reproduction rate (R_f = final population/initial population). Nematode population density was determined by extracting eggs and J_2s from soil samples (Coolen, 1979) and from the entire root system after exposure to 1% NaOCl for 5 min (Hussey & Barker, 1973). Severity of nematode galling of the root system was not assessed because no visible symptoms are caused by *M. artiellia* infection (Greco, 1984). The experiment was repeated once.

Effect of essential oils

This experiment was carried out under a controlled environment in a growth chamber adjusted to the conditions described above. Essential oil of *C. coronarium* flowerheads was added in four concentrations (10, 20, 30 and 40 μL per 500 cm^3 soil) by applying measured quantities of stock solution to four sterile cotton pellets distributed

regularly around the chickpea radicle at sowing. Control plants were treated similarly with sterile distilled water. Nematode inoculum and inoculation method were as previously described. There were six replicate plants for each essential oil concentration in a completely randomized design, each replicate consisting of a single potted plant. The experiment was repeated once.

Statistical analysis

Data were subjected to ANOVA using STATISTIX (NH Analytical Software, Roseville, MN, USA). Similarity between experimental runs, tested by preliminary ANOVAs using experimental runs as blocks, allowed the data to be combined for ANOVAs. In the hatching and mortality of J_2 test, the area under cumulative percentage hatch (AUCPH) and the area under cumulative percentage mortality (AUCPM) curves values were estimated by the trapezoidal integration method for each essential oil concentration (Campbell & Madden, 1990). Treatment means of AUCPH, AUCPM and final cumulative egg hatch and mortality of J_2 at each essential oil concentration, as well as treatment means of *in planta* experiments, were compared using Fisher's protected least significant difference test (LSD) at $P = 0{\cdot}05$. In the *in planta* experiments, all data on nematode population density (X) were transformed into $\log_{10}(X + 1)$ before analysis (Gomez & Gomez, 1984).

To describe the effects of essential oil concentrations (OC) on cumulative percentage hatch, AUCPH and reproduction rate, the expanded negative exponential model:

$$C \exp[-r \log(\mathrm{OC})] + K$$

was fitted to the data, where C is a constant, r is the rate of decrease over OC, and K is the asymptote. Similarly, to describe the effects of OC on cumulative percentage J_2 mortality and AUCPM, the monomolecular model:

$$K \{1{\cdot}0 - B \exp[-r \log(\mathrm{OC})]\}$$

was fitted to the data, where K is the asymptote, B is a constant, and r is the rate of increase over OC (Campbell & Madden, 1990). Regression analyses were conducted by the least-squares program for nonlinear models of the Statistical Analysis System 6·08 (SAS Institute Inc., Cary, NC, USA). The coefficient of determination (R^2), the mean square error, the asymptotic standard error associated with the estimated parameter, and the pattern of the standardized residuals plotted against either predicted values or the independent variable were used to evaluate the appropriateness of a model to describe the data (Campbell & Madden, 1990).

Results

In vitro experiments

Influence of essential oils on hatch

Percentage hatch of *M. artiellia* eggs was significantly influenced ($P < 0{\cdot}05$) by the concentration of essential oil from *C. coronarium* flowerheads throughout the experiment (Fig. 1a). Final percentage hatch, as well as AUCPH, was significantly reduced ($P < 0{\cdot}05$) in egg masses incubated at any of the essential oil concentrations tested (Fig. 1a). For final percentage hatch and AUCPH, there were no significant differences ($P = 0{\cdot}05$) between essential oil concentrations of 2 and 4, or between 8 and 16 μL mL^{-1}, with the minimum hatch values occurring with the two highest oil concentrations (Fig. 1a). Both cumulative percentage hatch and AUCPH decreased exponentially with increasing in essential oil concentration (Fig. 2).

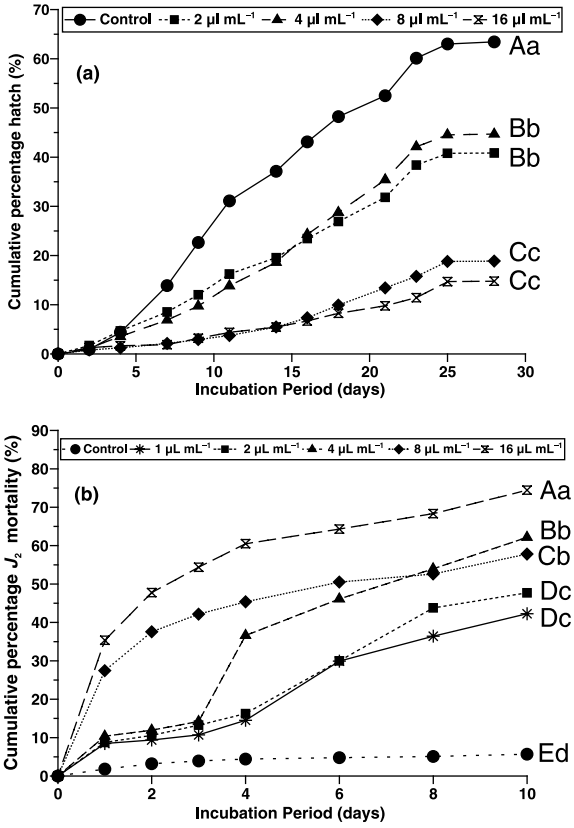

Figure 1 (a) Cumulative hatch of *Meloidogyne artiellia* over 4 weeks' incubation at 20°C in a series of concentrations of essential oil of *Chrysanthemum coronarium* flowerheads. (b) Cumulative mortality of J_2s of *M. artiellia* over 10 days' incubation at 20°C in a series of concentrations of essential oil of *C. coronarium* flowerheads. Each point represents the average of two experiments with five replicates. AUCPH of curves from each treatment combination or final hatch followed by the same upper-case or lower-case letters, respectively, do not differ ($P > 0{\cdot}05$) according to Fisher's protected LSD test.

Nematicidal activity of essential oils on J_2*s*

The viability of J_2s of *M. artiellia* was significantly influenced ($P < 0{\cdot}05$) by the concentration of *C. coronarium* essential oil throughout the experiment (Fig. 1b). Final J_2 mortality, as well as AUCPM, was significantly lower ($P < 0{\cdot}05$) in J_2s incubated in SDDW than in those incubated at any essential oil concentration (Fig. 1b). Percentage

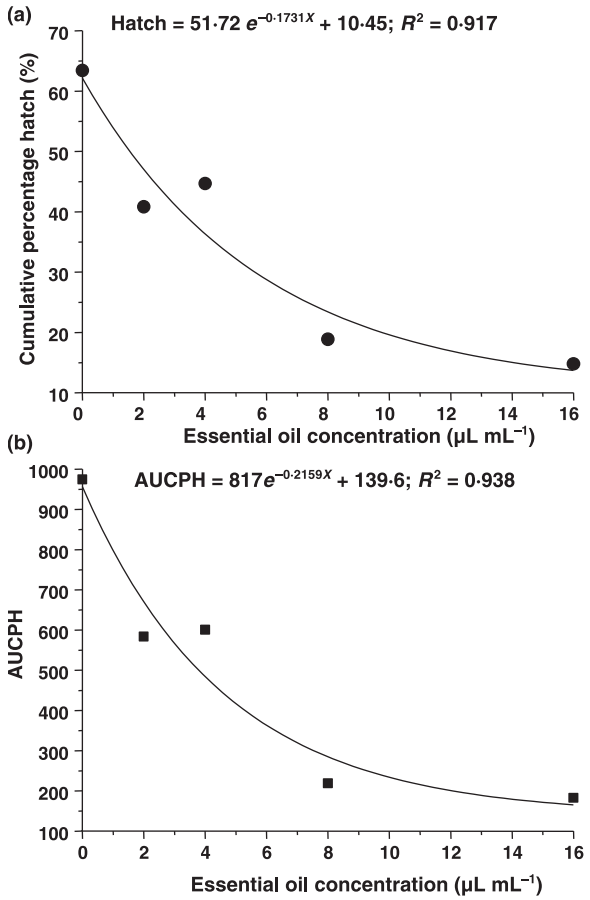

Figure 2 Relationship between *Chrysanthemum coronarium* essential oil concentration (2, 4, 8 or 16 μL mL^{-1}) and final cumulative hatch (a) or AUCPH (b) of *Meloidogyne artiellia* over 4 weeks' incubation at 20°C. Each point represents the average of two experiments with five replicates. The line represents the predicted function calculated by the expanded negative exponential model.

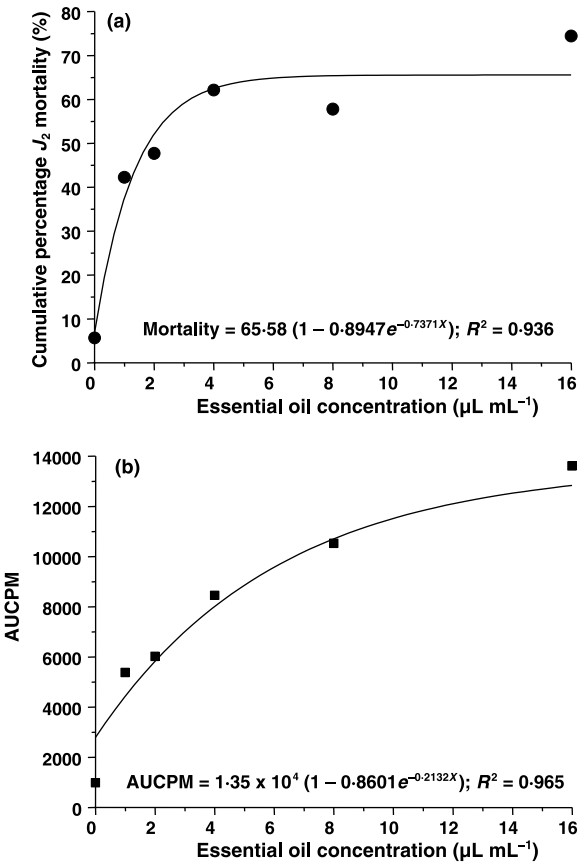

Figure 3 Relationship between *Chrysanthemum coronarium* essential oil concentration (1, 2, 4, 8 or 16 μL mL^{-1}) and final cumulative J_2 mortality (a) or AUCPM (b) of *Meloidogyne artiellia* over 10 days' incubation at 20°C. Each point represents the average of two experiments with five replicates. The line represents the predicted function calculated by the monomolecular model.

mortality and AUCPM were significantly higher ($P < 0{\cdot}05$) in J_2s incubated in essential oil at 16 μL mL^{-1}, the highest concentration tested, than at any other concentration (Fig. 1b). Cumulative percentage J_2 mortality and AUCPM showed a monomolecular type of increase with increasing essential oil concentration (Fig. 3).

In planta experiments

Effect of organic amendments

Growth of chickpea cv. PV 61 plants, determined by dry shoot and fresh root weights, was not affected by infection with *M. artiellia* in the absence of organic amendments (Table 2). Similarly, growth of chickpea plants infected by *M. artiellia* grown in soil with organic amendments from several Asteraceae was not significantly affected ($P = 0{\cdot}05$) by nematode infection, irrespective of plant part or species evaluated (Table 2). The incidence of *M. artiellia* infection of chickpea plants was not affected by any of the organic amendments, but the reproduction rate of *M. artiellia* was significantly reduced ($P < 0{\cdot}05$) by all the organic amendments, irrespective of plant species or part, when compared with the nonamended treatment (Table 2). The reproduction rate of *M. artiellia* was significantly less ($P < 0{\cdot}05$) in chickpea plants amended with *Calendula officinalis* and *Calendula suffruticosa* than in plants amended with leaves of *Chrysanthemum coronarium*. Minor differences were observed among the other treatments, which gave intermediate reproduction rates (Table 2).

Effect of essential oils

Essential oil concentrations of *C. coronarium* did not influence ($P > 0{\cdot}05$) the growth of chickpea cv. PV 61, determined by dry shoot and fresh root weights (Table 3). However, the reproduction rate of *M. artiellia* on chickpea cv. PV 61 was significantly ($P < 0{\cdot}05$) reduced by application of all concentrations of essential oil compared with untreated plants. The percentage reduction ranged from 38·0 to 54·1% of the reproduction rate reached in control plants. However, reproduction rate was not significantly ($P > 0{\cdot}05$) different between the concentrations of essential oil applied in this experiment.

Table 3 Influence of essential oil from *Chrysanthemum coronarium* on the growth of chickpea cv. PV 61 and reproduction of *Meloidogyne artiellia*[a]

Essential oil concentration (µL per 500 cm^3 soil)	Nematode inoculum (eggs + J_2 cm^{-3} soil)	Plant growth		R_f[b]
		Shoot DW	Root FW	(P_f/P_i)
0	0	1·17 a	12·76 a	0
0	20	1·21 a	14·20 a	52·8 a
10	20	1·05 a	14·61 a	32·8 b
20	20	1·13 a	16·09 a	31·3 b
30	20	1·24 a	15·61 a	29·5 b
40	20	1·13 a	12·16 a	24·3 b

[a]Data are the averages of two experiments with five replicated plants per treatment combination in each experiment. Means followed by the same letter do not differ significantly ($P > 0·05$) according to Fisher's protected LSD test.

[b]R_f (nematode reproduction rate) = final nematode density per plant /initial nematode population density per plant. Data were transformed to log ($X + 1$) for analysis.

Discussion

The suppressive effects of some phytochemical compounds on nematode populations has been well documented in several pathosystems (Chitwood, 2002). These compounds could be developed for use as nematicides themselves, or could serve as model compounds for the development of environmentally friendly synthetic derivatives. This study was designed to evaluate the nematicidal activity of the essential oil of *C. coronarium* flowerheads or organic amendments from several species of the family Asteraceae on the root-knot nematode *M. artiellia*. *In vitro* and *in planta* experiments clearly demonstrated that egg hatch, J_2 survival and reproduction rate of the nematode were significantly reduced by the essential oil of *C. coronarium* flowerheads. Similarly, treatment with each of several plant species and plant parts (flowers, leaves, roots and seeds of *C. coronarium*, and flowers of *Chrysanthemum segetum*, *Calendula officinalis*, *Calendula maritima and Calendula suffruticosa*) consistently reduced the reproduction rate of *M. artiellia* on chickpea compared to the nonamended treatment. The data for the nematicidal activity of Asteraceae agree with results of other researchers, who found that the population density of *Meloidogyne* spp. was reduced when host plants were grown in soil amended with *Chrysanthemum* spp. (Hackney & Dickerson, 1975; Tiyagi *et al*., 1988; Bélair & Benoit, 1996) or *Calendula* spp. (Tiyagi *et al*., 1988). However, the results of the present study regarding the effects of nematode parasitism on plant growth under artificial conditions are not in agreement with the results of other researchers under field conditions (Di Vito & Greco, 1988), who found a significant reduction in plant growth and seed yield. Differences in the susceptibility of the chickpea cultivars or differences in environmental conditions could be responsible for this.

Essential oils from several plant species have been shown to have nematicidal activity on root-knot nematodes *in vitro* and in soil (Chatterjee *et al*., 1982; Sangwan *et al*., 1985; Soler-Serratosa *et al*., 1996). Results from the present *in vitro* and *in planta* experiments indicated that essential oil compounds from *C. coronarium* flowerheads directly affect nematode biology by interfering with nematode hatching and J_2 viability, as reported for other phytochemical compounds (Chandravadana *et al*., 1994; Chitwood, 2002). The influence of the essential oil on egg hatch and J_2 survival of *M. artiellia* is probably related to its nematicidal activity against other *Meloidogyne* spp. Thus the data suggest that the essential oil from *C. coronarium* has potential to be used in nematode control. Additionally, as essential oils from *C. coronarium* have also been reported to have insecticidal and fungicidal activities (Pérez *et al*., 1999; Alvarez-Castellanos *et al*., 2001), treatment of soil with essential oils or their components could serve as a means of soil disinfection. However, the practical use of essential oils or organic amendments to control *M. artiellia* on chickpea has constraints: the direct costs associated with the cultivation of such plant species or their incorporation into soil. Further investigations are needed to confirm the nematicidal activity of these compounds on other root-knot nematodes affecting high-value crops (those produced or marketed at relatively greater risk or cost of production, such as nursery crops, fruits and vegetables) before they can be incorporated into an integrated pest management system.

In conclusion, these results suggest that species of Asteraceae may be used in crop rotations as antagonistic plants or green manures to reduce *Meloidogyne* populations. Dry preparations of these plants or extracts of the active principles could be used as nematicides.

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