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Nematicidal activity of *Chrysanthemum coronarium*

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Abstract

Organic amendments and green manure are potential alternatives to the harmful chemical control means currently used against plant-parasitic nematodes. In this work, *Chrysanthemum coronarium* was applied to the soil as a green manure to control the root-knot nematodes *Meloidogyne incognita* and *M. javanica*. *Chrysanthemum coronarium* significantly reduced nematode infection of tomato roots and improved plant-top fresh weight, both in the greenhouse and in microplots. Other green manures, derived from *Anthemis pseudocotula*, wild chickpea (*Cicer pinnatifidum*), *Geranium* spp. and wheat, were not as effective as *C. coronarium*. *Chrysanthemum coronarium*, retained its nematicidal activity even when applied as a dried material. Only mature *C. coronarium* plants, in their flowering stage, exhibited nematode control activity, but the green plant parts were more effective than the flowers. An aqueous extract of *C. coronarium* exhibited *in vitro*, nematostatic activity towards *M. incognita* and *M. javanica* second-stage juveniles and inhibited their hatching from eggs and egg-masses; its nematostatic activity was expressed also against other phytonematode species such as *Heterodera avenae* and *Pratylenchus mediterraneus*, but did not affect the beneficial entomopathogenic nematode *Steinernema feltiae*.

Introduction

Plant-parasitic nematodes are economically important pests of many crops worldwide, and the root-knot nematodes (*Meloiodogyne* spp.) cause a major share of these losses. At present, pathogenic nematodes are moderately controlled by agrotechnical, chemical and genetic (breeding) means (Manzanilla-Lopez et al., 2004). Use of all these means is fairly limited because of their high cost, incomplete efficacy and inadequate specificity. Often there are also severe risks involved in nematicide application: their residues pose environmental hazards such as toxicity to other soil organisms, with possible consequent disruption of the soil ecology. In addition, some residues may be hazardous to human health. Methyl bromide, one of the most commonly used and efficient biocides, will be banned very soon in the Middle East; its use is already forbidden in several countries in Europe. As a consequence, it is imperative that new approaches to nematode control be developed and applied (Manzanilla-Lopez et al., 2004). Possible alternatives to nematode management are the integration of antagonistic plants, biological control agents, green manures, natural products and organic amendments.

Antagonistic plants have been used as a source of nematicidal substances, which are isolated from various plant organs, usually by hydro- or organic distillation methods (Ferraz and de Freitas, 2004). The most reported antagonistic plants are *Tagetes* spp. (family Asteraceae), neem (*Azadirachta indica*, family Meliaceae), *Crotalaria juncea*, velvet bean

(*Mucuna* spp.), and several species of grasses from the Poaceae family (Chitwood, 2002; Ferraz and de Freitas, 2004). These plants have revealed broad activity against various soil-borne organisms, including those that cause plant diseases, such *Fusarium*, *Verticillium*, etc. (Ferraz and de Freitas, 2004).

Pérez et al. (2003) investigated the effects of essential oils, extracted from several species of Asteraceae, on *Meloidogyne artiellia*; the strongest nematicidal activity was exhibited by the essential oil obtained from the flower heads of *Chrysanthemum coronarium*. Flowers from five Asteraceae species, as well as various parts of *C. coronarium* (flowers, leaves, roots or seeds), which were applied as amendments to nematode-infected soil, also significantly reduced the reproduction rates of *M. artiellia*.

In the present study we aimed to expand the knowledge of the effect of *C. coronarium*, used as a green manure, and to compare its efficacy with that of other plant species that might be considered as green manure amendments against the root-knot nematode, *Meloidogyne javanica*, and against other phyto- and entomopathogenic nematode species. *In vitro* tests of aqueous extracts, as well as growth chamber and micro-plot experiments, were conducted to evaluate the nematicidal activity of various fresh and dried plant organs of *C. coronarium*.

Materials and methods

Nematode inocula

Populations of the root-knot nematodes *Meloidogyne incognita* and *M. javanica* were reared on susceptible tomato plants (*Lycopersicon esculentum* var. 144) in a greenhouse for two months. Eggs were separated from egg masses with sodium hypochlorite (0.5%, 1 min), and were used for inoculation of the soil in pot experiments. Second-stage pre-infected juveniles (J2) were hatched from the eggs in water for 24 h. In microplot experiments, the soil had been infested by growing nematode-infected tomato plants in it before the experiment. Tomato speed-seedlings cv. 144 (Hishtill Nursery, Israel) were used in all the greenhouse experiments; they were planted in the potting mixture.

Heterodera avenae (J2) and *Pratylenchus mediterraneus* (all life-stages) were obtained from a culture maintained on wheat plants grown in a screenhouse, and infective juveniles (IJ) of *Steinernema feltia*e were supplied by Dr. I. Glazer, of the Nematology Department at the Volcani Center.

Plant material

Chrysanthemum coronarium plants were harvested during spring (March and April, 2001 and 2002) from a wild field, at the ARO's Bet Dagan campus, Israel. Fresh plants were chopped and used as green manure. On several occasions, fresh shoots and leaves were separated from the flower heads before use in the experiments. To obtain dry material, the chopped plants or the separated plant organs (shoot plus leaves, and flower heads) were dried for 72 h at 70 °C, crumbled, and stored at room temperature.

Besides *C. coronarium*, various plants, belonging to several botanical families were also tested: *Anthemis pseudocotula*, chickpea (*Cicer pinnatifidum*), *Geranium* spp., safflower (*Carthamus tinctorius*), tomato (*L. esculentum* var. 144) and wheat (*Triticum aestivum* var. Bet Hashita). They were collected, chopped and used as green manure as described for *C. coronarium*, in parallel experiments at the same time.

C. coronarium *extract*

Fresh plants (10 g of shoots) were chopped and blended with 100 ml of distilled water for 10 min at room temperature. After separation of the crude plant extract, the supernatant solution was filtered on Whatman paper No. 1 and then on a 0.22 μm cellulose acetate filter (Corning Costar, NY).

In vivo *and* in vitro *nematostatic bioassays*

The bioassays were performed at room temperature, in 24-well polystyrene culture plates containing 500 μl per well, of aqueous extract of the plant, or sterilized distilled water as control. The following nematodes in the specified life stages were added to the wells: I. about 100 J2 of each of *M. incognita, M. javanica* and *Heterodera avena*e; 100 IJ of *Steinernema feltia*e (entomopathogenic nematode) and ca 100 species of *Pratylenchus mediterraneu*s (all life stages); II. about 100 separated eggs of

M. incognita or *M. javanica*; III. one egg mass of *M.incognita* or *M. javanica*. Each treatment was replicated in six wells. Following the first observation, after 48 h, the plant extract and the control solutions were removed and the contents of each well were re-suspended in 500 μl of distilled water. The percentages of immobilized J2s and of hatching eggs were determined with an inverted microscope (×40). These assays were performed three times.

Experiments were performed to distinguish nematicidal from nematostatic activity: The bioassays were performed at room temperature, in 24-well polystyrene culture plates containing 500 μl per well, of three concentrations (25, 12.5 and 6.25%) of aqueous extract of the plant, or sterilized distilled water as control. Two hundreds separated eggs of *M. javanica* were added to the wells. Each treatment was replicated in six wells. Following the first observation, after 72 h, the plant extract and the control solutions were removed and the contents of each well were re-suspended, for 72 h, in 500 μl of distilled water. The percentages of hatching eggs were determined with an inverted microscope (×40). These assays were performed three times.

To assess the observations of the *in vitro* bioassays, eight batches of about 1000 separated eggs of the *Meloidogyne* species, which had previously been exposed to 500 μl of plant extract or distilled water, for 72 h, were introduced into the 750 ml pots in which tomato seedlings were growing, in order to compare the infectivity of the *C. coronarium*-treated nematodes with that of the untreated ones. The plants were fertilized with a 20-20-20 nutrient solution (75 mg l^{-1}) and irrigated with plain tap water as needed. Thirty-five days after exposure of the seedlings to the nematodes, the plants were removed and their top fresh weights and root-galling indices were evaluated on a 0–5 scale (Bridge and Page, 1980).

Growth chamber experiments

Chopped fresh or dried plants of *C. coronarium* were mixed with sandy loam soil (pH 7.8; 0.8% organic matter) at a rate of 1 or 0.2% w/w, respectively (since fresh plants lose 80% of their weight in drying), in 750 ml pots. The soil in the pots had been pre-infested with 3000 nematode eggs or with 2000 J2 of *M. javanica* per pot, and the pots were placed in a temperature-controlled (27 ± 2 °C) growth-chamber. Ten days after the green manure had been added to the soil, tomato speed-seedlings cv. 144, purchased from the Hishtill nursery (Ashkelon, Israel), were planted. Untreated nematode-infested soils served as controls. The plants were fertilized and irrigated with plain tap water as needed. Thirty-five days after exposure of the seedlings to the nematodes, the plants were removed and their top fresh weights and root-galling indices were evaluated on a 0–5 scale. On some occasions, nematode reproduction was evaluated by extracting eggs from each root and counting the number of eggs per root. Each experiment was conducted three times, with 10 replicates per treatment.

Micro-plot experiments

The experiments were conducted during the spring and summer of 2002 and 2003. Each plot comprised a concrete cylinder of 1 m diam, buried in the soil to a depth of 2 m; each was naturally infested with *M. javanica*. The nematode infestation levels were mapped at 'time-zero', in order to ensure uniform distribution among the various subsequent treatments. Mapping was performed by randomly sampling each plot (six soil samples per plot), placing the samples in 300 ml pots, each sample in a pot, and planting nematode-susceptible test plants (tomato seedlings) in the pots. Four weeks later, the plants were harvested and the galling indices were determined on a 0–5 scale. Each treatment was applied in four plots, each planted with six tomato seedlings. The soil was amended with the *C. coronarium* by mixing the green manure to a depth of 50 cm: fresh material was applied at a rate of 0.5% (w/w) and the dried material at 0.1% (w/w). Untreated nematode-infested plots served as controls. One month after application of the green manure to the soil, tomato seedlings cv. 144 were planted. The plants were uprooted two months later, and shoot and fruit weights, and root galling indices on a 0–5 scale were recorded.

Characterization of the active compounds

The *C. coronarium* aqueous extract was separated into two fractions with a Centricon device fitted with a filter having a 3-kDa molecular weight (MW) cutoff (Amicon, Millipore Corporation, Bedford, MA, USA). The lower-MW fraction (<3 kDa) was used without further treatment in

the *in vitro* bioassay, described above, and the higher-MW fraction (>3 kDa), which was concentrated on the membrane, was re-suspended in distilled water with 5 mM $CaCl_2$. Nematostatic activity, after heating, was tested after boiling the *C. coronarium* aqueous extract solution for 10 min, in open or closed tubes, and running the *in vitro* assays mentioned above. Nematostatic activity at various pH ranges was tested by adding NaOH solutions of appropriate concentrations. This experiment was repeated three times.

Statistical analysis

Experiments were conducted at least twice. Data were subjected to ANOVA using Genstat V programme; means were comparing using Tukey's HSD test ($p \leq 0.05$).

Results

Evaluation of C. coronarium *and other plants as green manures*

Amendment of *M. javanica*-infested soil with *C. coronarium* green manure resulted in a most significant reduction of the galling of tomato roots as compared with the non-amended control and with other green manure amendments (*Geranium* sp., safflower, tomato and wheat), except of wild chickpea (*C. pinnatifidum*) and *A. pseudocotula* (Figure 1). The effects of the various green manures on the numbers of nematode eggs g^{-1} of tomato plant root exhibited the same trend: in the *C. coronarium* treatments, 1158 eggs g^{-1} were counted on the infected roots, as compared with 3858 and 4132 eggs g^{-1} in the safflower and wheat treatments, respectively, and in the chickpea and *Anthemis* treatments egg production was similar to that in the control (26,360; 36,650 and 29,750 eggs g^{-1}, respectively). In non-infested soil, the top fresh weights of tomato plants grown on *C. coronarium* pre-amended soil was significantly higher than that of tomatoes grown on soil without green manure (29.9 and 22.8 g $plant^{-1}$, respectively), whereas in *M. javanica*-infested soil, the top fresh weights of tomato plants grown on *C. coronarium*-treated soil was not significantly greater than that of plants grown on non-amended soil (25.7 and 21.8 g $plant^{-1}$, respectively).

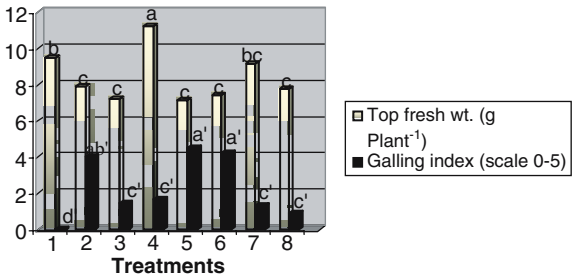

Figure 1. Effect of fresh green manure on tomato plants infected with *Meloidogyne javanica*. Experiment was performed in 10 replicates, in 750 ml pots containing sandy loam soil artificially infested with 2000 second-stage juveniles (J2) and amended with 2% (w/w) of green manure of: 1. *Chrysanthemum coronarium*; 2. Control (unamended-soil); 3. Tomato (*Lycopersicum esculentum* var. 144); 4. *Geranium* sp.; 5. Chickpea (*Cicer pinnatifidum*); 6. *Anthemis pseudocotula*; 7. Safflower (*Carthamus tinctorius*); 8. Wheat (*Triticum aestivum* var. Bet Hashita). Four weeks later, the pots were planted with tomato seedlings. Top fresh weights (grams $plant^{-1}$) and galling indices (on a 0–5 scale) of the infected roots were determined 35 days after planting. Means within columns followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's HSD test.

Application of C. coronarium *green manure at different rates*

Soil amended with *C. coronarium* green manure was effective against the root-knot nematode at all application rates tested (0.5–4% w/w). Whereas the top fresh weight was increased only with the higher application rates, i.e., 2 and 4%, a significant reduction in galling index was achieved with every application rate, although the greatest effect was found in the highest application rate treatment, i.e., 4% (w/w) (Table 1).

Application of dried C. coronarium

Dried chopped *C. coronarium* plants, at a rate equivalent to 2% (w/w) of fresh material, were very effective against the root-knot nematode (Table 2). Galling indices of tomato plants grown on *C. coronarium*-treated soil were significantly lower and their top fresh weights were significantly higher than those of tomato plants grown on untreated soil.

Effect of different plant parts of Chrysanthemum

Pre-mature (before flowering), 2–3 month-old *C. coronarium* plants, had no effect on galling

Table 1. Effects of *Chrysanthemum coronarium* green manure, applied at various rates, on tomato plants infected with *Meloidogyne javanica*

Treatment[1]	Top fresh wt[2, 3] (g plant^{-1})	Galling Index[2, 3] (0–5 scale)
Control	5.0 b	1.5 a
0.5% green manure	6.2 b	0.38 b
1% green manure	6.8 b	0.36 b
2% green manure	10.5 a	0.40 b
4% green manure	9.8 a	0.06 c

[1] Experiment was performed in 750 ml pots containing sandy loam soil artificially infested with 2000 second-stage juveniles (J2) and mixed with 10 replicates of *C. coronarium* amendments (0.5–4% w/w) at various rates. Nematode-infected soil served as control. Four weeks later, pots were planted with tomato seedlings.
[2] Top fresh weight and galling indices of the infected roots were determined 35 days after planting.
[3] Means within columns followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's HSD test.

Table 2. Effect of dried *Chrysanthemum coronarium* plants on tomato plants infected with *Meloidogyne javanica*

Treatment[1]	Top fresh wt.[2, 3](g plant^{-1})	Galling index[2, 3] (0–5 scale)
Control	7.0 b	4.1 a
Dried Chrysanthemum	10.3 a	0.5 b

[1] Experiment was performed in 750 ml pots containing sandy loam soil artificially infested with 3000 eggs of *M. javanica* and mixed with 10 replicates of 0.4% (w/w) dried material (equivalent to 2% fresh material); 10 days later, the pots were planted with tomato seedlings.
[2] Top fresh weight and galling indices of the infected roots were determined one month after planting.
[3] Means within columns followed by different letters were significantly different at $P \leq 0.05$ according to Tukey's HSD test.

indices of *M. javanica*-infected tomato roots. However, significantly greater top fresh weights were found in both nematode-infected and uninfected plants (data not shown). Green plant parts (stems and leaves) of *C. coronarium* were much more effective (lower galling indices and higher top fresh weights) than dried flowers (Table 3).

Application of fresh and dried C. coronarium *in micro-plots*

Use of *C. coronarium* in micro-plots showed a similar trend to that observed in pot experiments, i.e., fresh and dried *C. coronarium* plants were

Table 3. Effect of different parts of *Chrysanthemum coronarium* plants, applied as green manure amendment, on tomato plants infected with *Meloidogyne javanica*

Treatment [1]	Top fresh wt [2, 3] (g plant^{-1})	Galling index [2, 3] (0–5 scale)
control	7.0 b	4.1 a
C. coronarium (flowers)	6.6 b	2.3 b
C. coronarium (green parts)	10.3 a	0.5 c

[1] Experiment was performed in 750 ml pots containing sandy loam soil artificially infested with 2000 second-stage juveniles (J2) and mixed with 10 replicates of 2% (w/w) of *C. coronarium*; Nematode infected soil served as control. Four weeks later, pots were planted with tomato seedlings.
[2] Top fresh weight and galling indices of the infected roots were determined 35 days after planting.
[3] Means within columns followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's HSD test.

both equally effective in the reduction of nematode infection and in the improvement of top and fruit fresh weights of tomato plants (Table 4).

Nematostatic effect of C. coronarium *extracts on* M. incognita *and* M. javanica *life- stages*

Both *M. incognita* (data not shown) and *M. javanica* exhibited the same nematostatic reaction, *in vitro*, to *C. coronarium* extracts. J2 were immobilized, and hatching of eggs, either those in egg-masses or separated eggs, was inhibited (Table 5). The reversibility experiments performed with *M. javanica* revealed that the number of J2s hatched from eggs after 72 h exposure to the aqueous extract of *C. coronarium* was much lower as compared to that of J2s number hatched after transferring to washing in distilled water (Table 6). Moreover, hatching and recovery of the hatched J2s increased as the percentage of the aqueous extract decreased (Table 6). Inoculation of tomato seedlings with the treated eggs resulted in significantly lower galling indices than those obtained with the untreated control (0.5 and 2.7 respectively, on a 0–5 scale).

Nematostatic effect of C. coronarium *aqueous extract on different nematode species*

As with the *Meloidogyne* species, the J2 of *Heterodera avena*e were very much affected by *C. coronarium* aqueous extract, and *Pratylenchus*

Table 4. Effects of fresh or dried *Chrysanthemum coronarium*, applied as green manure amendment in micro-plots, on tomato plants infected with *Meloidogyne javanica*

Treatment[1]	Shoot fresh wt[2, 3] (g plant^{-1})	Fruit wt[2, 3] (g plant^{-1})	Galling index[2, 3](0–5 scale)
Control	263.3 b	509.3 b	3.5 a
C. coronarium (fresh)	688.0 a	1223.6 a	1.3 b
C. coronarium (dry)	647.3 a	1330.1 a	1.3 b

[1] Experiment was performed in microplots within naturally infested soil and mixed with 0.1% (w/w) dried material, equivalent to 0.5% fresh material.
[2] Top and fruit fresh weights and galling indices of the infected roots were determined 2 months after planting.
[3] Means within columns followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's HSD test.

Table 5. Nematostatic effects, after 48 h exposure, of *Chrysanthemum* coronarium aqueous extract on *Meloidogyne javanica* life stages [eggs and second-stage juveniles (J2)]

Treatment	Immobilized J2 (%)	Egg hatching (%)	No. of J2s hatched egg-mass^{-1}
Control	0	80	140
C. coronarium	93	0	0

*mediterraneu*s populations were partially immobilized. However, the entomopathogenic nematode *Steinernema feltia*e was not affected by the aqueous extract of *C. coronarium* (Table 7).

Characteristics of C. coronarium *aqueous extract activity*

Most activity of the aqueous extract was expressed in the low-MW fraction, below 3 kDa. The activity was heat resistant. Changing the pH from 5 to 6 lowered the activity by 50%, while at pH values of 7–9, activity was completely absent (data not shown).

Discussion

The nematicidal and nematostatic activities of *C. coronarium*, mainly against the root-knot nematode, *M. javanica*, and also against representatives of other plant- and entomo-parasitic species, were evaluated in *in vitro* and in-soil (*in vivo*) experiments. It has been generally known that application of green manure could enhance plant growth and increase plant tolerance to damage caused by nematodes (Ferraz and de Freitas, 2004), but our present results reveal that *C. coronarium* was effective even at rates that were too low to increase plant growth. The effects of soil amendment with *C. coronarium* applied as a dry material were similar to those obtained when it was applied as a fresh material, in both pot and micro-plot experiments. In practice, the application of fresh green manure might be impracticable, because the growing season of *C. coronarium* in Israel is only during the winter, with flowering around March–April (spring time); therefore, the finding that dried *C. coronarium* plant material is effective in nematode control is very important with regard to the possibility of using it in all seasons. Screening for nematicidal effects among Compositae plants revealed that *C. coronarium* had some effects on other plant-parasitic nematodes: Tiagi and Wani (1992) reported on reduced populations of the nematode *Tylenchorhynchus brassicae* in soils amended with *C. coronarium*; and the same effect was found against *M. incognita* and *Rotylenchulus reniformis* (Tiagi et al., 1988).

Further examination of the nematicidal effect of *C. coronarium* against root-knot nematodes, revealed that only mature, flowering *C. coronarium* plants, exhibited nematode control activity and application of different plant parts showed that shoots and leaves were more effective than the flowers. Antibacterial activity of methylene chloride extract of fresh *C. coronarium* flower heads was reported by Urzua and Mendoza (2003), and similarly, Pérez et al. (2003) reported that the nematicidal effect of *C. coronarium* against *M. artiellia* in chickpea was best expressed by the flowers. This activity was attributed to essential oils in the flower heads.

In the present study, aqueous extract of *C. coronarium* plants exhibited nematostatic activity against several life-stages (egg-masses, separated eggs and J2) of root-knot nematodes and the effect was irreversible: the extract-treated nematodes were incapable of infecting roots in soil. This *in vitro* activity was heat resistant and was attributed

Table 6. Nematostatic effects, after 72 h exposure, of different concentrations of *Chrysanthemum coronarium* aqueous extract on *Meloidogyne javanica* second-stage juveniles (J2) hatched from 200 eggs

Aqueous extract concentration (% of the original)	Average no. of J2s[1] hatched 200 eggs^{-1} (72 h after exposure to the extract)	Average no of J2s[1] hatched 200 eggs^{-1} (72 h after washing in water)
25.00	0.83 a	6.33 a
12.50	4.00 b	35.16 b
6.25	13.66 c	48.00 b
Control	80.16 d	97.30 c

[1] Means within columns followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's HSD test.

Table 7. Nematostatic effect, after 24 h exposure, of *Chrysanthemum coronarium* aqueous extract on different nematode species

Nematode	Immobilized nematodes (%)	
	Control	*Chrysanthemum*
Meloidogyne javanica	0	98
Heterodera avenae	3	93
Pratylenchus mediterraneus	5	56
Steinernema feltiae	7	0

to a substance of low molecular weight. Sharma and Trivedi (1991) screened plant extracts for nematicidal activity and found some *in vitro* effect of *C. indicum* leaf extract on *M. incognita* egg hatching. Preliminary tests revealed that the active component was probably a low-MW substance with acidic properties, as the nematostatic activity was eliminated at high pH.

Chrysanthemum coronarium and *Tagetes* spp. belongs to the family *Asteraceae*. According to Gommers and Bakker (1988), the mechanism responsible for the nematicidal activity of *Tagetes* is related to the generation of singlet oxygen (which is a general bio-toxin) by photoactivation of α-thertienyl which diffuses to the root. Whether the same mechanism acts in *C. coronarium* is a question for further investigation.

The *C. coronarium* extract showed nematostatic activity against other plant-parasitic nematodes, but had no effect on beneficial entomopathogenic nematodes. These results are very encouraging in that they suggest that *C. coronarium* can potentially be used against a broad range of plant-parasitic nematodes, without harming beneficial nematodes.

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