Journal of Nematology 35(4):437–442. 2003.

Effect of *Artemisia vulgaris* Rhizome Extracts on Hatching, Mortality, and Plant Infectivity of *Meloidogyne megadora*[1]

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Abstract: The activity of an ethanolic rhizome extract of *Artemisia vulgaris* against hatching, mortality, host plant infectivity, and galling of the root-knot nematode *Meloidogyne megadora* was investigated. The extract inhibited egg hatch (50% inhibition by 2.35 mg/ml) and caused second-stage juvenile mortality (50% lethality at 12 hours' exposure to 55.67 mg/ml), both in a dose-dependent manner. Nematode infectivity on *Phaseolus vulgaris* 'Bencanta Trepar', a susceptible host, decreased in a dose-responsive manner (50% inhibition at 6.28 hours exposure to extract). When applied directly to the soil, the extract reduced root galling on a susceptible host in a dose-dependent manner (50% inhibition by 32.36 mg/ml). After dilution in distilled water, the extract did not lose activity when stored in the dark at 25 °C for 15 days.

Key words: Artemisia vulgaris, botanical-nematicide, *Meloidogyne megadora,* mugwort, root-knot nematode, toxicity.

Representatives of the genus *Meloidogyne,* the root-knot nematodes, are among the most damaging nematodes in agriculture, causing an estimated $100 billion loss/year worldwide (Oka et al., 2000). *Meloidogyne megadora,* first found attacking coffee, *Coffea arabica,* in the Democratic Republic of S. Tomé and Príncipe, is a polyphagous plant-parasitic nematode with a wide host range (Abrantes et al., 1995; Almeida et al., 1997). Temperature requisites for *M. megadora* development (Almeida, unpubl.) indicate that Mediterranean countries could sustain populations of this species.

Nematicides are usually not available or not affordable in developing countries (Korayem et al., 1993), and the demand for alternative, safe, and inexpensive natural nematicides has promoted the screening of plants for antihelminthic activity (Chitwood, 1993). Strong nematotoxic activities against *M. incognita* have been detected in plants of the genus *Artemisia.* Aqueous extracts of fresh roots of *Artemisia absinthium* inhibited juvenile hatching (Sharma and Trivedi, 1992a), and soil treated with dried roots decreased root galling of *Solanum melongena* by *M. incognita* (Sharma and Trivedi, 1992b). Aqueous extracts of the dried plant shoots also inhibited juvenile mobility by 100% after 72 hours, and hatching by 98.7% after 30 days (Korayem et al., 1993). Aqueous extracts of dried aerial parts caused mortality in more than 90% of second-stage juveniles (J2) of *M. incognita* after a 24-hour exposure, as did similar extracts of *A. verlotorum* (Dias et al., 2000). Infested soil treated with chloroform extracts of dried foliage decreased the number of juveniles in soil in 2 weeks. Similar extracts of *A. cina* and *A. dracunculus* produced analogous effects (Abivardi, 1971). Aqueous extracts of *A. annua* and *A. pallens* fresh shoots induced 100% mortality in 12 hours and prevented hatching of juveniles of *M. incognita* (Pandey, 1990). Activity against *M. javanica* J2 was also reported in aqueous extracts of dried leaves of *A. argyi* and of dried seedlings of *A. capillaris* (Ferris and Zheng, 1999).

Artemisia vulgaris (mugwort) is one of seven *Artemisia* species occurring in Portugal (Franco, 1984), and its activity against root-knot nematodes has not been previously investigated. However, polyacetylenes extracted from *A. vulgaris* caused 100% mortality of *Caenorhabditis elegans* adults after photoactivation by UV radiation, and are insecticidal (Wat et al., 1981).

The objectives of the present study were to assess the activity of a hydrophilic ethanolic rhizome extract of mugwort on hatching, mortality, plant infectivity, and plant damage of *M. megadora* with a goal of identifying novel leads for nematicides, or for use as soil amendments.

Materials and Methods

Extraction of plant components: The mugwort plants were collected in Mesio, Castro Daire, Portugal. Rhizomes were washed thoroughly in running tap water and then cut into small pieces that were dried at a temperature not exceeding 45 °C. They were then ground to a fine powder using a coffee mill. Twenty-five grams of this powder (corresponding to ca. 175 g wet weight) was suspended in 200 ml of ethanol-water (1:1) in a 500-ml flask for 48 hours in the dark on an orbital shaker at 200 rpm. The suspension was filtered under vacuum with Whatman No. 1 filter paper, the residue re-extracted for an additional 48 hours, and similarly filtered. The two filtrates were combined, evaporated to dryness in a rotatory evaporator at 45 °C, and the resulting extract (ca. 5 g) was stored at 5 °C until use.

Nematode population: The *M. megadora* population, obtained from naturally infected coffee plants from the Democratic Republic of S. Tomé and Príncipe, was maintained and increased on a susceptible host, *Phaseolus vulgaris* 'Bencanta Trepar' (Almeida et al., 1997).

Received for publication 21 May 2002.

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The authors thank Alípio Dias & Irmão for supplying seeds; J. Morgado, Ervital, for supplying full-grown *Artemisia vulgaris* plants; and R. Inserra (Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Nematology Bureau) and K. Evans (IACR-Rothamsted) for their critical review of the manuscript.

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This paper was edited by Keith Davies.

Hatching bioassay: The extract was dissolved in distilled water to obtain final concentrations of 0.5, 1, 6.25, 12.5, 25, 50, and 100 mg/ml, and the solutions were filtered 3 times with Whatman No. 1 filter paper. Egg masses were picked from roots infected with *M. megadora,* transferred to distilled water, and torn with a needle to release the eggs, from which those embryonated with J2 were handpicked using a bristle. Each treatment consisted of four replicates of 15 eggs in 0.4 ml of each extract concentration. The experiments were conducted in a glass-staining block maintained in a moist chamber, in the dark, at 25 °C. Hatched J2 were counted after 3, 7, and 15 days in treatments and controls (distilled water).

To determine whether extract activity was altered with time, testing with two of the extract concentrations, 1.0 and 12.5 mg/ml, was required to achieve detectable variations in extract activity. Extracts were replaced with fresh solutions after 3, 7, and 14 days as were distilled water controls.

Data on hatching were converted to percentage cumulative hatching inhibition, corrected by Abbott's formula (Abbott, 1925), and subjected to probit analysis (Finney, 1971). Data corrected only by Abbott's formula were further compared with those of 1.0- and 12.5-mg/ml extract concentrations of the hatching bioassay itself by ANOVA, followed by Duncan's multiple-range test using STATISTICA v. 5.0 for Windows (Statsoft, Tulsa, OK).

Mortality bioassay: Extract solutions of 25, 50, and 100 mg/ml were prepared as above. Second-stage juveniles, obtained from egg masses picked from infected bean roots, were placed in a 300-µm-pore muslin sieve in a petri dish (5 cm) containing distilled water at 25 °C. Twenty-four hours later, the resulting J2 suspension was discarded and those that hatched in the subsequent 24 hours were used for the bioassay. They were handpicked into 100 µl of distilled water on glass slides using a bristle and checked for normal movement using a stereomicroscope. Fifteen J2 were then transferred to each glass-staining block containing 0.4 ml of each extract solution; distilled water served as control. A second control tested the possibility of activity from solvent residues: 1.6 ml of distilled water poured into a dilution flask from which 200 ml of ethanol-water (1:1) had been evaporated to dryness. Each treatment was replicated four times. This bioassay was conducted in the dark, in a moist chamber, at room temperature (22 °C). Observations were made at 1, 3, 6, 9, 12, 18, 20, 22, 24, and 26 hours. Second-stage juveniles that did not move when touched with a bristle were transferred to distilled water. They were considered dead if they still failed to react to probing with a bristle one hour later.

Mortality data were converted to percentage cumulative mortality and corrected by Abbott's formula (Abbott, 1925) with reference to the distilled water control. Data derived from the 12-hour observation were subjected to probit analysis (Finney, 1971).

Infectivity bioassay: The infectivity bioassay, adapted from De Viala et al. (1998), employed bean plants grown in 100-cm^3 plastic pots filled with a mixture of autoclaved sand:autoclaved soil (1:1). One hundred recently hatched J2 were handpicked into 100 µl distilled water on a glass slide using a bristle. Then, 100 µl extract at 50 mg/ml was added to obtain a 25-mg/ml concentration, and to standardize time of exposure for all J2. The J2 were incubated in a moist chamber in the dark at room temperature for 1, 6, 12, or 24 hours. Second-stage juveniles exposed in this way were transferred to distilled water immediately before they were inoculated near the roots of a bean plant with two pairs of true leaves. Thus, plants were never in contact with the extract. Each exposure treatment consisted of five replicates, as did the unexposed control.

The pots were placed at randomly assigned positions in a growth room at temperatures ranging from 22 to 30 °C, and maintained under a 14/10-hour light/dark regime for 7 days. They were watered daily and fertilized once with Algoflash (N:P:K 6:6:6) water-soluble fertilizer (Algochimie, Chateau Renault, France). The plants were then uprooted, and their roots were washed free of soil and stained with acid fuchsin (Byrd et al., 1983). Nematodes in the entire root system of each plant were counted using a stereomicroscope, and infectivity was expressed as a percentage of the number that infected roots of control plants.

Split-pot bioassay: The split-pot bioassay was adapted from Alphey et al. (1988), using plastic boxes (3×7×5.2 cm), each divided by a 50-µm muslin sieve, firmly attached with waterproof tape. Each half was filled with 35 ml dry autoclaved sand:autoclaved soil (3:1), which had been washed through sieves to eliminate particles larger than 1 mm and smaller than 100-µm diam. A seedling of the susceptible cucumber *Cucumis sativus* 'Inglês Comprido' was planted in one side of the box, and 5 ml extract was added. One hundred recently hatched *M. megadora* J2, handpicked into 200 µl water on a glass slide using a bristle, were then transferred in 5 ml distilled water to the nonplanted side of the box. Three extract concentrations, 25, 50, and 100 mg/ml, were tested, with distilled water as a control. Each treatment was replicated 10 times. The boxes were randomly arranged in a chamber at 25 °C, 70% RH, under a 12/12-hour light/dark regime and were watered when needed. After 15 days, the boxes were split in their two halves, and nematodes in soil were collected from both the planted and the nonplanted sides through washing and sieving through a set of 70-µm and 25-µm sieves and counted from the resulting suspension. Plant damage was assessed by counting the number of galls per root system. The roots of five plants from each treatment were stained with acid fuchsin (Byrd et al., 1983)

TABLE 1. Effect of ethanol-water (1:1) extract of *Artemisia vulgaris* rhizome on *Meloidogyne megadora* J2 hatching inhibition (corrected cumulative percentages).

Extract concentration (mg/ml)	Cumulative hatching inhibition (%) after different exposure periods (days)[a]		
	3	7	15
0.5	5.1	11.1	13.0
1.0	47.6	23.1	24.1
6.25	69.2	71.1	71.7
12.5	85.7	94.2	94.3
25.0	90.9	95.7	96.3
50.0	100.0	100.0	100.0
100.0	100.0	100.0	100.0

Percentages are average of four replicates.
[a] Hatching inhibition = (percentage hatching inhibition in extract – percentage hatching inhibition in control) × 100 % / (100 – percentage hatching inhibition in control) (Abbott, 1925).

TABLE 2. Effect of ethanol-water (1:1) extract of *Artemisia vulgaris* rhizome, with and without replacement, on cumulative hatching inhibition of J2 of *Meloidogyne megadora* (actual values).

Extract concentration (mg/ml)	Cumulative hatching inhibition after 360 hours exposure[a]	
Replacement of extract solution		
1.0	1.51 ± 0.60	A
12.5	14.02 ± 0.97	B
No replacement of extract solution		
1.0	0.52 ± 0.18	A
12.5	13.51 ± 0.91	B

Data are means of four replicates ± SD. Values followed by the same letter are not different according to Duncan's multiple-range test ($P < 0.05$).
[a] Hatching inhibition = (hatching inhibition in extract-hatching inhibition in control) × 15 / (15-hatching inhibition in control) (Abbott, 1925).

and examined using a stereomicroscope to count nematodes that had infected the root.

Data on root galling was converted to percent galling inhibition, corrected using Abbott's formula (Abbott, 1925) and subjected to probit analysis (Finney, 1971).

RESULTS

Hatching bioassay: All seven extract concentrations inhibited J2 hatching at each exposure period (Table 1); hatching of control nematodes was 89.6 ± 8.8%. Generally, inhibition of hatching gradually increased with time and was dose-dependent. Exposure to 0.5 mg/ml extract caused least inhibition of J2 hatching, and 50- and 100-mg/ml extracts produced 100% inhibition at all times. The probit plot of corrected percentage cumulative inhibition against extract concentration displays a typical dose-responsive effect (Fig. 1); the extract concentration inhibiting hatching by 50% (IC_{50}) after 360 hours exposure was 2.35 mg/ml. Data from the hatching bioassay in which the treatment solutions were replaced were not different from equivalent data in the first bioassay ($P < 0.05$) (Table 2).

Mortality bioassay: The mortality in the solvent residue control was not different from the mortality in the distilled water control. One-hundred-percent mortality was achieved by exposure to the three extract solutions, although it occurred after different times of exposure. The 100-mg/ml extract induced 100% mortality within a 9-hour exposure, whereas the 50-mg/ml extract elicited the same effect in 22 hours and the 25-mg/ml extract in 26 hours (Fig. 2). Second-stage juvenile mortality was dose dependent with an LC_{50} in a 12-hour period of 55.67 mg/ml (Fig. 3).

Infectivity bioassay: The 25-mg/ml extract inhibited the infectivity of exposed *M. megadora* J2 on a susceptible host (Fig. 4), with infectivity decreased by 35% after exposure periods as short as 1 hour. A 6-hour exposure of J2 to this extract concentration decreased the number of nematodes inside roots by 57%, and a 70% decrease occurred with a 12-hour exposure period. The greatest observed effect on infectivity was an 84% decrease after a 24-hour exposure period. The exposure time for a 50% decrease of infectivity was estimated as 6.28 hours.

Split-pot bioassay: Total nematode recovery from planted and nonplanted soil, after 15 days, ranged from

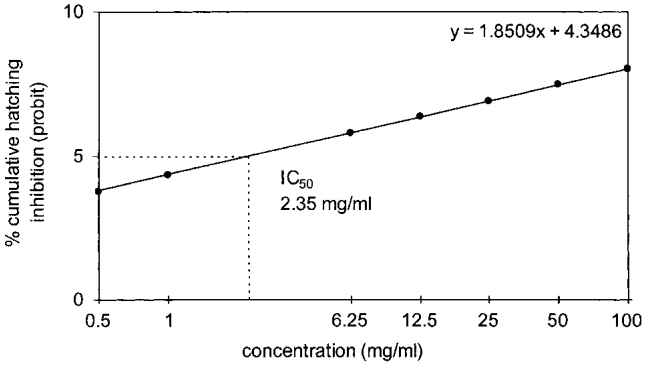

FIG. 1. Probit plot of the effect of exposure for 360 hours to ethanol-water (1:1) extract of *Artemisia vulgaris* rhizome on *Meloidogyne megadora* J2 hatching.

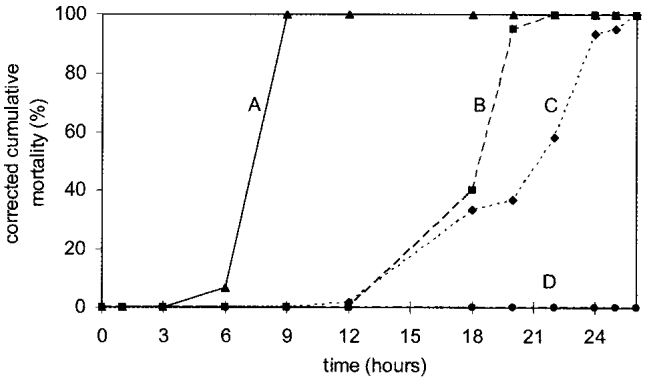

FIG. 2. Corrected percentage cumulative mortality of *Meloidogyne megadora* J2 exposed to different concentrations of ethanol-water (1:1) extract of *Artemisia vulgaris* rhizome, and to the possible solvent residue. A) 100-mg/ml extract. B) 50-mg/ml extract. C) 25-mg/ml extract. D) solvent residue.

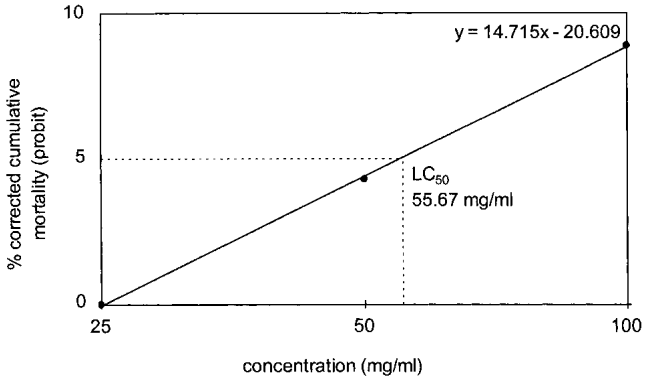

FIG. 3. Probit plot of the effect of exposure for 12 hours to ethanol-water (1:1) extract of *Artemisia vulgaris* rhizome on *Meloidogyne megadora* J2 mortality.

58.6% in the control to 10.4% after exposure to the 100-mg/ml extract (Table 3). Percentages of nematodes found inside roots, relative to the total nematode recovery, were 97% and 40% in the control and treatment, respectively. The percentage of nematodes recovered from soil in planted and nonplanted sides of the box increased with increasing extract concentrations. Fewer nematodes were recovered from the planted side than from the nonplanted side after exposure to any extract concentration tested, in contrast to control, from which more nematodes were recovered from the planted side. Percentages of nematodes in roots and total nematode recovery after exposure to the extracts were different from those of the control ($P < 0.05$) (Table 3). Root galling, was reduced in a dose-dependent manner (Fig. 5), with an IC_{50} after 15 days of 32.36 mg/ml.

DISCUSSION

The 100% hatching inhibition induced by the 50-mg/ml preparation of the extract is as high as any reported for extracts of other *Artemisia* species; water extracts of fresh shoots of *A. annua* and of *A. pallens* inhibited hatching of *M. incognita* by approximately 99.6% and 99%, respectively, at an extract concentration of 333 mg/ml (Pandey, 1990). This is about six

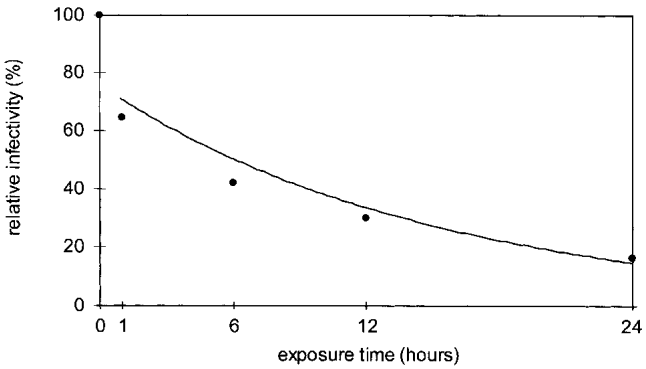

FIG. 4. Infectivity of *Meloidogyne megadora* on bean cv. Bencanta Trepar after exposure to ethanol-water (1:1) extract of the *Artemisia vulgaris* rhizome at 25 mg/ml.

TABLE 3. Mean numbers (%) of *Meloidogyne megadora* recovered from planted and non-planted sides of the box, after 15 days' exposure to an ethanol-water (1:1) extract of *Artemisia vulgaris* rhizome.

Extract concentration (mg/ml)	J2 in nonplanted soil[a]	J2 in planted soil[a]	Nematodes in plant roots[b]		Total nematodes recovered[b]	
0	0.8	0.9	57.0 ± 6.3	A	58.6 ± 6.2	A
25	1.9	0.2	19.2 ± 11.8	B	21.3 ± 11.6	B
50	4.6	0.9	14.8 ± 6.2	B	17.5 ± 5.6	BC
100	3.7	2.6	4.2 ± 2.6	C	10.4 ± 2.3	C

[a] Percentages are average of 10 replicates.
[b] Percentages are average of five replicates ± SD. Values followed by the same letter are not different according to Duncan's multiple-range test ($P < 0.05$).

times more concentrated than our extract. A water extract of dried shoot of *A. absinthium* induced 98.7% hatching inhibition of *M. incognita* at 50 mg/ml (Korayem et al., 1993), a concentration similar to the data presented here. Only one plant species has previously been studied in regard to hatching inhibition of *M. megadora.* A dichloromethane extract (0.1 mg/ml) of *Xanthosoma sagittifolium* produced 85% hatching inhibition after 28 days' exposure (Galhano et al., 1998). Corrected hatching inhibition (Abbott, 1925) may vary along exposure time, reflecting not only the extract activity but also the influence of hatching in control.

The nematicidal effect of the extract was dose-dependent. As the mortality bioassay was an acute toxicity test, the extract solutions were not replaced as in the hatching bioassay. It is assumed, however, that no extract degradation occurred during the 26 hours of the test. As residues from the solvent did not kill J2, nematotoxicity is attributed to the extract constituents.

The extract was more active than the fresh shoot water extracts of *A. annua* and of *A. pallens,* which caused 100% mortality of *M. incognita* J2 at 12 hours only at a concentration of 167 mg/ml (Pandey, 1990). It was also more effective than a dried shoot water extract of *A. absinthium,* which inhibited *M. incognita* J2 activity by 100% only after 72 hours of exposure (Korayem et al., 1993). Our results are similar to those ob-

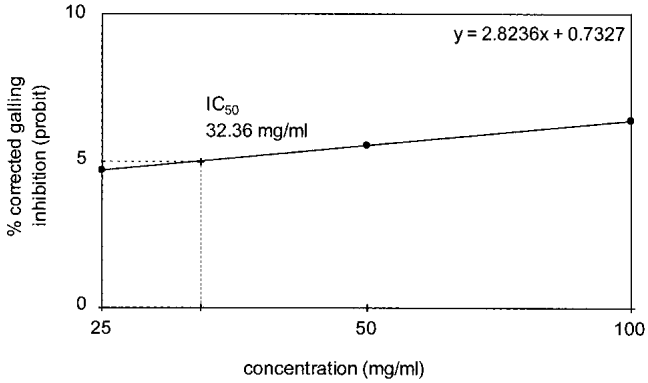

FIG. 5. Probit plot of the effect of exposure for 15 days to ethanol-water (1:1) extract of *Artemisia vulgaris* rhizome on plant damage by *Meloidogyne megadora* J2.

tained with aqueous extracts of dry aerial parts of *A. absinthium* and of *A. verlotorum* that cause more than 90% mortality of *M. incognita* J2 at 100 mg/ml, after a 24-hour exposure (Dias et al., 2000). Other plants previously screened for lethal activity against *M. megadora* J2 are *Colocasia esculenta* and *X. sagittifolium*. Dried corm dichloromethane extracts of these plants took at least a 12-hour exposure period to cause mortality (Galhano et al., 1997; 1998), compared to the 100% mortality after 9 hours' exposure to the 100-mg/ml concentration of our extract.

Polyacetylenes extracted from various plants, including *A. vulgaris,* have caused 100% mortality of adults of *Caenorhabditis elegans* at 0.5 mg/ml, but activity was dependent on polyacetylene activation by UV radiation (Wat et al., 1981). As all of our extractions and bioassays were protected from light, deterioration in extract activity by exposure to light was not relevant.

Few studies have assessed the effects of plant extracts or compounds on plant infection by nematodes. From our results we estimated a 50% decrease in infectivity after 6.28 hours of exposure of *M. megadora* J2 to 25 mg/ml ethanolic rhizome extract of *A. vulgaris*. A 4-hour exposure of *M. incognita* J2 to a 20-ppm solution of thiarubrine C, isolated from roots of *Rudbeckia hirta,* resulted in no infectivity of tomato *Lycopersicon esculentum* cv. Rutgers (De Viala et al., 1998). However, a true comparison of activity will be possible only after isolation and purification of active compounds from the *A. vulgaris* extract, as ethanol-water extraction of *A. vulgaris* produces a very complex mixture of compounds (Olah et al., 1998). The observed gradual decrease in infectivity with increasing exposure time is probably due to *M. megadora* J2 becoming less mobile or vigorous after exposure to nematotoxic compounds in the extract. The 25-mg/ml extract solution caused mortality only after a 12-hour exposure, so the decreased infectivity within that exposure time may have been due to a J2 behavioral change at a concentration below the lethal range. There is a possibility that lethal effects would have been observed eventually if J2 were transferred to water after short exposure periods. Behavioral changes at sublethal concentrations are usually an indicator of neurotoxicity (Williams and Dusenbery, 1990). Infectivity testing could be a useful tool in determining the mode of action of active compounds in the extract. Also, the protocol of exposing the J2 to the test extract in solution, followed by a test of infectivity, may serve as a laboratory first approximation of what would occur after a drench treatment; active extracts or compounds could then be subjected to pot bioassays.

In the split-pot bioassay, percentage of recovery of J2 after exposure to the extract was low. As J2 mortality was dependent on exposure to the extract, nonrecovered nematodes were presumably dead and disintegrated. Such low J2 recovery impaired further analyses of repellency and (or) feeding deterrency. However, the relatively higher percentage of recovered J2 in soil in the inoculated side after a 15-day exposure suggests that the extract interfered with nematode mobility and orientation. This is supported by the apparent behavioral change induced by a sublethal exposure to the 25-mg/ml extract in the infectivity test. Root galling was reduced in a dose-dependent manner. There was no evidence of phytotoxicity.

Probit analysis has helped in the interpretation of the present results and may encourage the use of dose-response curve analysis of screening data for comparative purposes.

The present results also seem sufficient to warrant studies on purification and the mode of action of bioactive compounds of *A. vulgaris,* and on the efficacy of other extracts on this and other species of plant-parasitic nematodes. Bioassay-guided isolation of active compounds along with complementary tests such as microplot experiments will help us clarify the means of exploiting *A. vulgaris* nematotoxicity in order to develop inexpensive, natural, and environmentally friendly nematicides.

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