EFFECTS OF *MELIA AZEDARACH* ON *MELOIDOGYNE INCOGNITA IN VITRO* AND *IN VIVO* CONDITIONS

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Received: *26 January 2012; Accepted*:*29 May 2012.*

Summary. Different concentrations of aqueous extracts (0%, 1%, 2% and 4%) of dried leaves, seed, seed kernels and seed coats of *Melia azedarach* L. were tested for their effect on motility and mortality of second stage juveniles (J2s) of *Meloidogyne incognita in vitro*. Also, in glasshouse conditions, the effects of powder preparations (0.0, 0.02, 0.04 and 0.08 w/w) of the same plant materials were examined on nematode activity and plant growth components of tomato (*Solanum lycopersicum* L.). The extracts of all plant parts and at every concentration immobilized 100% of J2s. Maximum (100%) J2 mortalities were given by concentrations of 2% and 4% of seed kernel and 4% of whole seed. No J2 mortality was observed at 1% water extract concentration of leaves. In the glasshouse, all treatments significantly reduced numbers of galls, egg masses and eggs in tomato roots, J2s in soil and tomato roots and final nematode population. Rates of 0.04 and 0.08 w/w of the seed kernel and of 0.08 w/w of the seed coat powders completely controlled the nematode in tomato roots. All treatments significantly ($P<0.01$) reduced tomato root length and shoot length in comparison to the non-infested and non-treated control.

Key words: Persian lilac, plant powders, plant aqueous extracts, *Solanum lycopersicum*, tomato.

A wide range of crops have been reported to be affected by root knot nematodes (*Meloidogyne* spp.), which occur world wide and can cause severe yield losses (Barker *et al*., 1976; Dasgupta and Gaur, 1986; Sasanelli, 1994; Gill and Jain, 1995). The use of chemical nematicides creates a hazard potential for humans, animals and the environment (Tsay *et al*., 2004). For these reasons, several plant materials have been investigated to assess their pesticidal properties. Linford *et al.* (1938) were the first to study the nematicidal effect of chopped pine-apple (*Annanas comosus* L.) leaves used as an organic amendment against *Meloidogyne* spp., while a review of phytochemical strategies for the control of nematodes was given by Chitwood (2002).

Among different medicinal plants that have pesticidal effects, scientists have focused on the family Meliaceae since the middle of the 20th century, when the pesticidal effects of the neem tree (*Azadirachta indica* L.) was recognized in India (Singh and Sitaramaiah, 1967, 1970). *Melia azedarach* L. belongs to the family Meliaceae (synonyms: *Melia australis* Sweet, *Melia candollei* Sw., *Melia japonica* G. Don, *Melia sempervirens* Sw.). It is commonly referred to as Persian lilac, white cedar, Chinaberry or bead tree, lunumidella and Ceylon cedar; it grows mainly in the north of Iran, but recently has also been grown in warm and tropical regions of the country. Azadirachtin is the common active nematicidal ingredient in most species of the Meliaceae family (Chitwood, 2002).

Before the present research work, no investigation had been conducted on the nematicidal effects of *M. azedarach* in Iran. However, many articles had been published on the nematicidal properties of this plant in other countries (Lee, 1990; Akhtar and Mahmood, 1993, 1994; Abd-Elgawad and Omer, 1995; El-Nagdi and Mansour, 2003; Cristobal-Alejo *et al*., 2006; Ntalli *et al*., 2010b; Katooli *et al*., 2010; Cavoski *et al*., 2012). Because of the extensive use of chemical nematicides such as methyl bromide in Iran, especially in greenhouses, that are hazardous to human and animal health and to the environment, the aim of this research was to assess the nematicidal effects of *M. azedarach* on *Meloidogyne incognita* (Kofoid *et* White) Chitw.

Therefore, the effects of aqueous extracts of dried leaves, seeds, seed kernels and seed coat of *M. azedarach* on motility and mortality of second stage juveniles (J2) of *M. incognita* were investigated under laboratory conditions. Also, the effects of 0.0, 0.02, 0.04 and 0.08 w/w of powder preparations of the same materials on nematode activity and tomato (*Solanum lycopersicum* L.) cv. Orobata plant growth were investigated in the glasshouse.

MATERIALS AND METHODS

Nematode population. The inoculum of *M. incognita* was initially isolated from infected tomato roots, identified and then multiplied in a greenhouse in 20 cm diameter pots containing sterile moist loamy soil (80% sand, 15% silt, and 5% clay) for 2 months at 25 ± 2 °C. Heavily infected tomato roots were washed free of adhering soil and used for preparing the inoculum. Nematode egg masses were carefully separated from the in-

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fected roots, put in Petri dishes in distilled water, and stored at 4 °C before use. Then the egg masses were incubated in a growth cabinet at 25 ± 2 °C to encourage emergence of second-stage juveniles (J2). The emerged J2s were collected daily from the Petri dishes for up to 5 days and used for the experiments.

Plant materials. Plant materials of *M. azedarach* were obtained from north Iran in September 2010 and dried in the shade. Different plant parts were then finely ground to powder and aqueous extracts and powders of leaves, seeds, seed kernels and seed coats were prepared to evaluate their nematostatic or nematicidal effect on *M. incognita*.

Experiment in vitro with plant aqueous extracts. Eight grams of the powders of each plant part were put in a double layered muslin cloth and macerated in 100 ml of distilled water for 12 h at 28 °C (8% w/v). The suspensions were then filtered, put separately in dark glass bottles and stored in a refrigerator at 5 °C until use. These suspensions were used as standard concentrations (8%) for each plant material and diluted with the appropriate amount of distilled water to obtain 1%, 2% and 4% (w/v) concentrations as reported in Table I.

The experiment was arranged in a completely randomized design with three replicates per treatment, for 24 h at 28 °C. Two hundred J2s per replicate in five millilitres of the tested solutions were poured in 3-cm-diameter Petri dishes. Then the numbers of non-motile J2 (nematodes which were not moving even after stimulation and therefore assumed to be dead) were counted under a stereomicroscope by observing 100 J2s randomly. Nematodes were then transferred to distilled water for a further 24 h before again counting to record the percentages of dead nematodes after transfer to fresh water.

Experiment in vivo with plant powders. Twenty-cm-diameter pots were filled with 2 kg of a mixture of autoclaved loamy soil mixed with plant material powder at the rate of 0.0% (control), 0.02%, 0.04% and 0.08% w/w. Pots were inoculated with freshly hatched *M. incognita* J2s at the rate of 2 J2/cm^3 soil and irrigated to allow plant material decomposition and the release of the chemical compounds present in the plant powders. The experiment was arranged in a completely randomized design with three replicates per treatments in a greenhouse at 28 °C under natural daylight length conditions. Two-weeks-old tomato seedlings were then transplanted into the pots five days after soil treatment. Two months later, nematode and plant growth variables, numbers of galls, egg masses, eggs, J2s per gram of root, J2s per gram of soil, final nematode population density and tomato stem and root lengths were recorded.

Second stage juveniles of the nematode were extracted from 100 g soil per pot, using the modified Baermann's funnel method (Timmer and Davis, 1982), and counted. The numbers of adult mature females in 1 g samples of feeder roots were assessed by staining the roots with acid fuchsin-lactophenol for 3 minutes and counting the females directly with the aid of a stereomicroscope. The egg masses per g of roots were counted directly under a stereomicroscope and separated from the roots. To count eggs, the egg masses were dissolved in 200 ml of 1.5% NaOCl aqueous solution by shaking for 4 minutes (Hussey and Barker, 1973). The resulting egg suspension was made up to a volume of 500 ml, thoroughly mixed and the eggs in three 1 ml sub-samples counted.

Roots were carefully washed free of soil, chopped

Table I. Different concentrations of plant materials and nematodes added.

Amount of standard (8%) concentration (ml)	Amount of distilled water (ml)	Amount of nematode suspension (ml)	Number of J2/ ml of nematode suspension	Final concentration (%)
5	0	5	40	4
2.5	2.5	5	40	2
2	6	8	40	1
0	5	5	40	0

Table II. Effect of different concentrations of aqueous extracts of *Melia azedarach* on the mortality of second stage juveniles of *Meloidogyne incognita*.

Aqueous extract concentration (%)	Mortality of second stage juveniles caused by			
	Leaf	Seed	Seed kernel	Seed coat
0.0	0.0 b*	0.0 c	0.0 c	0.0 b
1	0.0 b	2.0 c	4.3 b	4.3 b
2	9.3 b	78.0 b	100.0 a	92.3 a
4	25.3 a	100.0 a	100.0 a	94.3 a

*Data are means of 3 replicates. Data followed in each column by the same letters are not significantly different according to Duncan's Multiple Range Test (P = 0.05).

Table III. Analysis of variance of nematode mortalities caused by different parts of *M. azedarach*.

Subject of study	Mean square	F value	Probability	Coefficient of variation %
Leaf	0.17	12.90	0.0020	0.29
Seed	7988.00	87.80	0.0000	21.20
Seed kernel	9580.80	8843.80	0.0000	2.04
Seed coat	8291.80	115.70	0.0000	17.76

and a 5 g sub-sample per plant was boiled in lactophenol containing 0.1% acid fuchsin for 3 minutes and then placed in water in a food blender jar. They were then comminuted for two successive 15-s periods (Southey, 1986) and the nematode suspension was sieved through a 74-µm sieve to remove root debris. Numbers of J2s collected on a 63-µm sieve were counted and expressed per g of fresh root tissue.

Stem and root lengths of tomatoes were measured by using a ruler.

Statistical analysis. The General Linear Model was used to perform the analysis of variance (ANOVA) using the SPSS 16 and MSTAT-C for Windows computer software package (SPSS Inc. Chicago, USA). Duncan's Multiple Range Test was used to compare means at P = 0.05 (Duncan, 1955).

RESULTS

Experiment *in vitro* with plant extracts

Aqueous extracts of all plant parts immobilized nematode J2s. One hundred per cent J2 mortalities were observed at concentrations of 2% and 4% of seed kernels and 4% of seeds; 4% and 2% preparations of the seed coats resulted in 94% and 92% mortality respectively, with no significant differences between them. Low nematode mortalities were observed at 1% preparations of seeds (2.0%), seed kernels and seed coats (both 4.3%). With aqueous extracts of leaves, no J2 mortality occurred at 1%, but 2% and 4% of this extract caused 9.3% and 25.3% J2 mortality, respectively (Table II).

Experiment *in vivo* with plant powders

Effects on the nematode. Galls, egg masses, eggs, J2

Table IV. Effect of different concentrations of *M. azedarach* powder on *M. incognita* and growth components of tomato plants, 60 days after inoculation.

Treatment		Galls/g of root	Egg masses/g of root	Eggs/g of root	Larvae/g of root	Larvae/100 g of soil	Total nematode population	Tomato stem length	Tomato root length
Plant part powder	%								
Leaf	0.02	7.7 b*	3.7 b	1019.3b	80.0 b	51.0 b	3411.0 b	25.7 bc	14.0 b
	0.04	5.7 bc	1.7 b	423.3 bc	46.3 bc	34.0 bc	1184.7 cd	29.3 bc	13.0 b
	0.08	1.3 de	0.7 b	160.0 c	12.3 cd	6.0 d	159.0 d	21.7 bc	12.0 b
Whole seed	0.02	5.0 bcd	2.3 b	643.3 bc	53.7 bc	33.3 bc	2075.3 bc	19.0 bc	12.3 b
	0.04	2.0 cde	1.0 b	255.0 c	20.0 cd	12.3 cd	780.7 cd	27.0 bc	16.7 b
	0.08	2.7 cde	1.7 b	410.3 bc	37.0 bcd	13.7 cd	828.3 cd	17.3 bc	12.0 b
Seed kernel	0.02	2.7 cde	1.0 b	282.0 c	26.3 cd	16.0 cd	853.7 cd	22.3 bc	13.0 b
	0.04	0.0 e	0.0 b	0.0 c	0.0 d	0.0 d	0.0 d	33.0 ab	14.0 b
	0.08	0.0 e	0.0 b	0.0 c	0.0 d	0.0 d	0.0 d	13.5 c	12.7 b
Seed coat	0.02	1.7 de	0.7 b	165.7 c	19.3 cd	10.0 cd	421.0 cd	20.0 bc	17.0 b
	0.04	1.0 e	0.0 b	0.0 c	11.0 cd	5.0 d	62.7 d	17.7 bc	14.7 b
	0.08	0.0 e	0.0 b	0.0 c	0.0 d	0.0 d	0.0 d	26.0 bc	19.3 b
Infested and non-treated control	0.0	27.3 a	23.7 a	6435.0 a	280.7 a	210.0 a	74561.0 a	21.0 bc	10.3 b
Non-infested and non-treated control (Healthy plants)	0.0	0.0 e	0.0 b	0.0 c	0.0 d	0.0 d	0.0 d	46.3 a	28.3 a

*Data are means of 3 replicates. Data followed in each column by the same letters are not significantly different according to Duncan's Multiple Range Test (P = 0.05).

per gram of root, J2 per gram of soil and final *M. incognita* population density were significantly reduced by all tested *M. azaderach* parts at the different rates (Tables IV and V). Generally, the highest concentration of each material achieved the highest percentage reduction of nematodes both in soil and roots.

The greatest effect on the nematode was achieved by treatments with 0.04 and 0.08 (w/w) of seed kernels powder, which completely controlled the nematode. Good nematode control was also achieved with powders of seed coats, seeds and leaves.

Non-significant differences were observed among the different treatments in the number of *M. incognita* egg masses. Similar results were observed with number of eggs and final nematode population densities, except in treatments with leaf powder at 2%. However, the nematode population was significantly ($P<0.01$) reduced by all plant powders in comparison with the infested but untreated control.

Powders of seed kernels and seed coats gave the greatest reduction in gall numbers, J2 per g of roots and J2 per g of soil as well as final nematode population, followed by seed and leaf powder treatments, which were statically different from the previous group of treatments.

Effect on plant growth components. No significant differences in tomato root length were observed among treatments with all tested plant parts and the infested but untreated control (Table IV). The greatest root length (28.3 cm) was observed in the second control (non-infested and non-treated), followed by treatments with 0.08% seed coats (19.3 cm) and 0.04% of whole seeds (16.7 cm). The shortest root length was recorded for tomatoes in the infested and non-treated control pots (10.3 cm). Root lengths in all other treatments, including the infested control, were significantly shorter than that observed in the healthy plants (non-infested control).

No significant differences were observed in tomato stem length, excepted in pots that received 8% of seed kernels (13.5 cm) and the uninfested control (46.3 cm), which recorded the least and the greatest stem lengths, respectively.

DISCUSSION

Results obtained *in vitro* showed that all non-motile nematodes resumed motility after they were transferred to fresh water. This would indicate that, at the tested concentrations, the water extracts of *M. azedarach* have highly nematostatic rather than nematicidal effect on J2s of *M. incognita*.

Based on data shown in Tables II and IV, it can be stated that the impact of extracts or powders of *M. azedarach* on nematode mortality, infection and reproduction rate depended upon their rates of application. When the concentration of the extract or the amount of plant powder was increased, nematode infection and reproduction rate decreased. These results agree with those obtained by Lee (1990), Akhtar and Mahmood (1993, 1994), Abd-Elgawad and Omer (1995), El-Nagdi and Mansour (2003), Hosseininejad (2004), Cristobal-Alejo *et al.* (2006), Ardakani *et al.* (2009), Ntalli *et al.* (2010b), Katooli *et al.* (2010), Ardakani (2011), and more recently by Cavoski *et al.* (2012).

The tested plant powders and extracts of *M. azedarach* were highly effective against *M. incognita*. As in other plants of the family Meliaceae, tetranortriterpenoids probably constitute an important toxic principle in *M. azedarach*. Tetranortriterpenoids are chemically related to Azadirachtin, the primary insecticidal compound in the commercially important Neem (*Azadirachta indica*) oil (Khan *et al.*, 1974; Devakumar *et al.*, 1985, 1986; Maile, 1995; Kraus *et al.*, 1993).

Ntalli *et al.* (2010a) and Cavoski *et al.* (2012) reported that furfural was the most active bio-nematicidal compound of the natural components of *M. azedarach*. Bhattacharya and Goswami (1987) also reported that among the several chemical constituents present in neem kernels, the limonoids have highly nematicidal effects. Also,

Table V. Analysis of variance of effects of different concentration of *M. azedarach* powder on *M. incognita* and growth components of tomato plants.

Variable	Mean square	F value	Probability	Coefficient of variation %
Galls/g of root	0.06	55.29	0.0000	0.11
Egg masses/g of root	0.03	25.2903	0.0000	0.11
Eggs/g of root	593.99	25.13	0.0000	12.55
Larvae/g of root	3.49	25.90	0.0000	1.14
Larvae/100g of soil	2.03	43.77	0.0000	0.67
Total nematode population	12070.67	130.45	0.0000	16.93
Tomato stem length	0.05	2.53	0.0193	0.43
Tomato root length	0.02	2.23	0.0366	0.26

the nematicidal effect of the tested treatments may possibly be attributed to their high contents of certain oxygenated compounds, which are characterized by their lipophilic properties that enable them to dissolve the cytoplasmic membrane of nematode cells and their functional groups, interfering with the enzyme protein structure (Knoblock *et al*., 1989). The mechanisms of plant extract actions may also include denaturing and degrading of proteins, inhibition of enzymes and interfering with the electron flow in the respiratory chain or with ADP phosphorylation (Konstantopoulou *et al*., 1994).

In general, the water extracts were less effective than plant powders against the nematode. We could not find any clear reason for this, but it could be due to natural conditions or to the slow and continuous release of the nematicidal compounds from plant powders in the soil. The difference could also be due to the continuous exposure of J2s to the nematicidal compounds in the soil. However, the results from the pot experiment confirmed those of the *in vitro* experiment and showed that the efficacy of seed materials at controlling *M. incognita* was greater than that of leaves.

As the nematicidal effects of whole seeds, seed kernels and seed coats were similar, we suggest that the extract or powder of whole seeds can be used for controlling the nematode. The separation of seed kernel and seed coat of *M. azedarach* is very difficult compared to that of *Azadirachta indica* (Ardakani *et al*., 2009; Ardakani, 2011). Greater nematotoxic potential of seeds of *Sesbania sesban* L., *M. azedarach*, *Calotropis procera* R. Br. and *Ricinus communis* L., than that of leaf extracts, was also reported by Lee (1990).

Ntalli *et al*. (2010b) reported that doses of melia methanol extract higher than 0.08% were nematicidal, whereas lower concentrations were nematostatic (loss of motility as a result of the presence of the substance was reversible). In a pot experiment, doses of melia methanol extract higher than 2.5% w/w caused 100% *M. incognita* control with an EC_{50} value of 0.916% w/w.

In our study, the effect on growth reduction of tomatoes was observed at the highest rate of melia seed kernel powder (0.08 w/w), but not at 0.02 and 0.04 w/w. Therefore, we do not recommend the use of large doses of the melia seed materials for field or greenhouse application.

Comparison between the infected and non-treated control and the non-infected and non-treated control (healthy plants) showed that 55% and 65% reductions in tomato stem and root lengths, respectively, were due to *M. incognita*.

Shifting from the dangerous use of synthetic nematicides to alternative nematode control methods is highly desirable, especially in developing countries where control relies mainly on chemical nematicides. Therefore, our results appear very promising as the use of natural or composted products, biological control agents and new eco-compatible control methods have many advantages such as reduction of production cost, easy and safe application and no negative impact on the environment (Sasanelli *et al*., 2008; Renco *et al*., 2009, 2010; Maistrello *et al*., 2010; D'Addabbo *et al*., 2011).

ACKNOWLEDGEMENTS

We are grateful to Dr. N. Greco and Professor H.S. Gaur for checking the manuscript, Dr. Reza Amiri Fahliani for data analysis, and the Department of Plant Protection, Agriculture and Natural Resources Research Center, Kohgilouyeh and BoyerAhmad province, Iran, for financial support.

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