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Bio-Nematicidal Effect of *Moringa oleifera* on Citrus Nematode *Tylenchulus semipenetrans*

Iqra Naeem[1], Anam Moosa[2]*, Faisal Zulfiqar[3], Waqas Ahmed Khan[1], Tahir Mahmood[4], Asmaa A. Hamad[5] and Hassan Alsberi

[1]*Department of Plant Pathology, University of Agriculture, Faisalabad, P.O. Box 38040, Pakistan*
[2]*Department of Plant Pathology, Faculty of Agriculture and Environment, The Islamia University of Bahawalpur, Bahawalpur, Pakistan*
[3]*Department of Horticultural Sciences, Faculty of Agriculture and Environment, The Islamia University of Bahawalpur, Bahawalpur, Pakistan*
[4]*State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China*
[5]*Department of Biology, College of Science, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia*

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Key words
***Citrus jambhiri*, Rough lemon, *Moringa oleifera*, Invasion and development, Plant biomass**

ABSTRACT

Tylenchulus semipenetrans is an economically important pest of citrus crops. In this study, the *in vitro* and *in vivo* effect of methanolic, ethanolic, and aqueous *M. oleifera* extracts was tested on the development of *T. semipenetrans*. The effect of *M. oleifera* extract on juvenile mortality was assessed at 24, 48, and 72 h intervals at thee different concentrations (S, S/2, S/4). Fresh leaf methanolic extract produced the highest *in vitro* juvenile mortality at "S" concentration after 72 h. Ethanolic *M. oleifera* extract showed the highest reduction in invasion and development of *T. semipenetrans* on *Citrus jambhiri* at 1, 6, and 12 weeks. The plant biomass including plant height, root length, and the number of leaves were also increased. At week 1 only the J1 stage, at week 6 J3 and J4 stages, while, at week 12 J4 stage were observed, immature and mature females were present. *In vivo*, the protective and curative effect of *M. oleifera* extracts on reproductive parameters and plant growth revealed that protective application was more effective to reduce the reproduction of *T. semipenetrans* and improved plant growth. Protective application of methanolic extract showed the highest reduction in reproductive parameters; juveniles/100mL soil, females/g root, and juveniles/g root, and enhanced plant height, root length, and the number of leaves. Therefore, it can be concluded that *M. oleifera* can be used to control *T. semipenetrans*.

INTRODUCTION

Citrus is the leading fruit crop of Pakistan. It is highly nutritive, and rich in Vitamin A and C, organic acids, sugars, amino acids, and carotenoids (Cohn and Duncan, 1990). Several biotic and abiotic factors have contributed to the low yield and poor quality of citrus in Pakistan. *Tylenchulus semipenetrans* is one of the most prevalent pests of citrus which is associated with the citrus slow decline (CSD) and severely limits the yield (Pervez *et al*., 2003). CSD is characterized by poor root development, and abnormal or sick appearance of plants with significantly low yield (Iqbal *et al*., 2006; Khanzada *et al*., 2008). It mainly invades though young feeder roots and the infection is aggravated by limited water availability and soil stress (Duncan and Noling, 1987).

Several management strategies have been used to control this nematode (Nasir *et al*., 2021). Among them the chemical pesticides are most widely used. However, nematicides are expensive and toxic to the environment (Wang *et al*., 2002). Moreover, the pathogens may develop resistance to the chemicals (Okigbo and Nmeka, 2004; Carvalho, 2004). The use of bio-pesticides of botanical origin is an eco-friendly way to manage CSD (Siddiqui *et al*., 2005). Botanical pesticides are biodegradable and least toxic to human health and the environment. Several antifungal plant extracts have been tested for their nematicidal effect against nematodes (Khan *et al*., 2019;

* Corresponding author: anammoosa1@outlook.com
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Aminisarteshnizi, 2021).

Moringa oleifera Lam (Moringaceae) is native to the western and sub-Himalayan region, India, Pakistan, Asia, Africa, and Arabia, cultivated in the Philippines, Cambodia, Central, North and South America, and Caribbean Islands (Morton, 1991). It is a fast-growing and drought-resistant deciduous tree. *M. oleifera* leaves and seeds possess antimicrobial properties against several pests of fruit crops (Fahey *et al*., 2005). *M. oleifera* contains antimicrobial compounds such as saponin, tannins, alkaloids, phenols, steroids, and reducing sugars. These phytochemicals have bio-nematicidal potential (Izuogu and Oyedunmade, 2009). *M. oleifera* extract reduced nematode population in mung bean, cowpea, and urdbean and also enhanced plant yield (Devi, 2002). Similarly, Murslain et al. (2014) recorded significant mortality of *M. javanica* on eggplant by the application of *M. oleifera* in combination with *Trichoderma harzianum*. Claudius-Cole *et al*. (2010) and Izuogu *et al*. (2013) also recorded the mortality of *M. incognita* in cowpea and maize, respectively by *M. oleifera* extracts. *M. oleifera* boosts crop yield by decreasing the nematode population.

The project was undertaken to evaluate the nematicidal efficacy of *M. oleifera* extracts to cause mortality and reducing the invasion and development of *T. semipenetrans* in citrus.

MATERIALS AND METHODS

T. semipenetrans *culture*

T. semipenetrans was isolated from root and soil samples collected from infected citrus trees following Whitehead and Hemming tray method (Whitehead and Hemming, 1965) and Baermann funnel method (McKenry and Roberts, 1985). *T. semipenetrans* was mass cultured on *Citrus jambhiri* (Rough Lemon). Six-week-old citrus seedlings were transferred to earthen pots containing sterilized sandy loam soil. The plants were inoculated with 1000 J_2/pot of *T. semipenetrans* and kept in the greenhouse. The plants were watered once a week.

In vitro *effect of* M. oleifera *extracts on juvenile mortality*

M. oleifera sample was taken from the botanical garden University of Agriculture, Faisalabad, Pakistan. Leaves were washed, surface sterilized with sodium hypochlorite and 95% absolute ethanol. The leaves were oven-dried at 70 ºC for 2 days. To prepare fresh and dry leaf aqueous extracts 10g of fresh and dry leaves were ground separately in 100mL distilled water, while, methanolic extracts and ethanolic extracts were prepared by grinding 10g of fresh and dry leaves in methanol and ethanol (Adegbite and Adesiyan, 2005). The extracts were filtered though Whatman No. 1 filter paper. The initial concentration was 10% and it was maintained as standard concentration "S". The S concentration was diluted up to 25% and 50% and maintained as S/2 and S/4 concentrations, respectively. The mortality of *T. semipenetran*s was assessed by pouring 5mL botanical extract from each concentration, S, S/2, and S/4 in sterilized Petri dishes 5cm in diameter. *T. semipenetrans* inoculum 1mL containing 25 freshly hatched J2's was pipetted out in the Petri dishes. Control treatment contained only 1mL nematode inoculum suspension. The Petri dishes were incubated at 25-28 ºC. Each treatment consisted of thee replicates. The juvenile mortality was assessed at 24, 48, and 72 h. Juvenile mortality was calculated by the formula given:

$$\text{Juvenile mortality (\%)} = \frac{\text{Number of dead juveniles}}{\text{Total number of juveniles}} \times 100$$

Effect of M. oleifera *extracts on invasion and development of* T. semipenetrans

Air-dried sieved soil was treated with "S" concentration of *M. oleifera* extract and distributed into 250 mL volume sterilized plastic pots. The soil was distributed in the pots and after 2 days 6-weeks old citrus plants were transplanted into these pots and kept in a growth chamber at 25 ºC for 12 weeks. In the control treatment, the soil was kept untreated. After 1 week of transplanting the plants were inoculated with 1000 freshly hatched J2's of *T. semipenetran*s. The experiment consisted of a set of 4 treatments and each treatment was replicated five times. Data on the development stages of *T. semipenetrans* was recorded at 1, 6, and 12-week. The total number of J2, J3, J4, immature females and mature females of *T. semipenetrans* were recorded.

In vivo *effect of protective and curative application of* M. oleifera *extracts*

To study the *in vivo* protective and curative effect of *M. oleifera* extracts on the invasion and development of *T. semipenetrants* six-months old citrus plants were transferred to earthen pots containing sterilized soil. The most effective concentration of aqueous, methanolic, and ethanolic *M. oleifera* extract from *in vitro* study was selected for this study. In the case of protective treatment 50mL, *M. oleifera* extract was sprayed on each plant though a calibrated sprayer. Plants were inoculated with freshly hatched 1000 J2's of *T. semipenetrans*. The inoculum was directly applied to the soil around the root zone of the plants. In the case of curative treatment 50mL *M. oleifera* extract was sprayed one day after inoculation with 1000 freshly hatched J2s of *T. semipenetrans*. The plants were kept in the greenhouse at 25 ºC for 8 weeks. Reproduction parameters of *T. semipenetrans* number of

juveniles/g root system, number of females/g root system, and number of juveniles/100 mL soil were recorded by following the formula given below:

$$\text{Relative density of nematode (\%)} = \frac{\text{Number of samples with } T.\ semipenetrans}{\text{Total number of samples}} \times 100$$

Plant growth parameters plant height (cm), number of leaves, and root length (cm) were also recorded after 8 weeks.

Statistical analysis

The experimental design was completely randomized. The data were subjected to Tukey's highly significant difference test (HSD) after ANOVA analysis. Data were analyzed using the statistical software Statistix (Ver, 8.1). Significant interactions were used to interpret the results.

RESULTS

Effect on juvenile mortality

In vitro, the effect of aqueous, methanolic, and ethanolic fresh and dry leaf extract of *M.* juvenile on juvenile mortality of *T. semipenetrans* indicated that plant extract treatment, concentration, time and their interaction had a highly significant effect on juvenile mortality (Table I). Both fresh and dry leaf extracts of *M. oleifera* showed highly significant juvenile mortality of *T. semipenetrans*. Extracts from all types of media ethanol, methanol, or water showed complete juvenile mortality of *T. semipenetrans* after 72-h. The lowest juvenile mortality was observed at 24-h which increased significantly after 72-h. The highest mortality was produced at "S" concentration, while, mortality was least by diluting the "S" concentration up to 50% "S/4" (Table II).

Table I. Interaction effect of *M. oleifera* extracts, their concentration and time of exposure on *in vitro* juvenile mortality (%) of *T. semipenetrans*.

Source of variation	Juvenile mortality (%)	
	Fresh leaf extract	Dry leaf extract
Plant extract (P)	***	***
Concentration (C)	***	***
Time (T)	***	***
Interaction		
P×C	***	***
P×T	***	***
C×T	***	***
P×C×T	***	***

*, significant, **, ***, highly significant, ns=non-significant at P=0.05, ANOVA.

Table II. Effect of *M. oleifera* extracts on *in vitro* Juvenile mortality (%) of *T. semipenetrans*.

Treatment/ Concentration	Time (Hours)	Juvenile mortality (%)	
		Fresh leaf extract	Dry leaf extract
Methanolic extract			
S	24	67.2hi	62.2h
	48	80.6e	83.5d
	72	100.0a	98.5a
S/2	24	55.2m	51.5j
	48	63.9j	57.2i
	72	95.2b	83.2d
S/4	24	44.9o	37.5n
	48	50.5n	46.2m
	72	91.8c	89.7c
Ethanolic extract			
S	24	73.5g	67.2f
	48	89.5d	83.2d
	72	100.0a	96.7ab
S/2	24	59.9k	55.7i
	48	66.5hi	64.9fg
	72	99.2a	95.7b
S/4	24	53.5m	48.9kl
	48	68.0h	63.2gh
	72	93.7b	84.8d
Aqueous extract			
S	24	55.9l	51.2jk
	48	77.3f	73.8e
	72	100.0a	98.5a
S/2	24	46.5o	34.5o
	48	65.5ij	61.0h
	72	99.9a	96.8ab
S/4	24	22.5q	23.4q
	48	33.5p	30.2p
	72	50.0n	47.5lm
Control			
	24	0.0r	0.0r
	48	0.0r	0.0r
	72	0.0r	0.0r

Values are mean of 5 replicates. Values in the columns with same letters are not significantly different from each other, analyzed by Tukey's HSD test at P=0.05.

Effect on invasion and development of T. semipenetrans

The interaction of plant extracts and weeks on invasion and development had a highly significant effect

on all developmental stages of *T. semipenetrans* J2, J3, J4, immature females, and mature females. Plant extracts, weeks, and their interaction affected the invasion and development of *T. semipenetrans* (Table III). Ethanolic *M. oleifera* extract showed the lowest invasion of all developmental stages of *T. semipenetrans* J2, J3, J4, mature females, and immature females. At week 1, only the J2 stage was observed. At week 6, J3 and J4 stages were observed, while, at week 12 J3, immature females and mature females were observed (Table IV).

Table III. Interaction effect of *M. oleifera* extracts and weeks on invasion and development of *T. semipenetrans*.

Source of variation	J2	J3	J4	Immature females	Mature females
Plant extracts (P)	***	***	***	***	***
Weeks (W)	***	***	***	***	***
Interaction					
P×W	***	***	***	***	***

For statistical details, see Table I.

Table IV. Effect of *M. oleifera* extracts and weeks on invasion and development of *T. semipenetrans*.

Treatment/ Week	J2	J3	J4	Immature females	Mature females
Methanolic extract					
1	132.7b	--	--	--	--
6	--	35.3c	162.7b	--	--
12	--	--	25.0ef	56.0c	179.0c
Ethanolic extract					
1	105.7d	--	--	--	--
6	--	23.0d	120c	--	--
12	--	--	21.0f	30.0d	139.0d
Aqueous extract					
1	116.3c	--	--	--	--
6	--	62.7b	155.7b	--	--
12	--	--	29.3e	70.7b	187.7b
Control					
1	279.3a	--	--	--	--
6	--	95.0a	263.0a	--	--
12	--	--	38.0d	92.3a	214.7a

For statistical details, see Table II.

Effect on growth parameters of rough lemon C. jambhiri

Growth parameters of plant height, root length, and the number of leaves were also taken after checking the effect of *M. oleifera* extracts on the invasion and development of *T. semipenetrans*. Methanolic *M. oleifera* extract showed maximum plant height, root length, and the number of leaves after control, while, infected control showed the lowest growth of *C. jambhiri* (Table V).

Table V. Effect of *M. oleifera* extracts on growth parameters of rough lemon *C. jambhiri*.

Treatment	Plant height (cm)	Root length (cm)	No. of leaves
Methanolic extract	16.2b	3.1b	19.5b
Ethanolic extract	13.5c	2.9bc	17.6c
Aqueous extract	10.8d	2.3cd	14.2d
Healthy control	18.8a	4.3a	23.3a
Infected control	9.09e	2.1d	11.8e

For statistical details, see Table II.

Protective vs curative effect on growth parameters of rough lemon C. jambhiri *and reproductive parameters of* T. semipenetrans

In vivo the interaction effect of curative and protective application of *M. oleifera* extracts on growth and reproductive parameters was assessed. Plant extracts and type of application affected plant height, root length, number of leaves, juveniles/100mL of soil, juveniles/g root, and females/g root, while, their interaction affected the number of leaves, juveniles/100mL soil, and females/g root. The interaction of treatment and time did not affect plant height, root length, and juveniles/g root (Table VI).

Table VI. Interaction effect of protective vs curative effect of *M. oleifera* extracts on growth parameters of rough lemon *C. jambhiri* and reproductive parameters of *T. semipenetrans*.

Source of variation	Plant height	Root length	No. of leaves	Juveniles/ 100mL soil	Females/ g root	Juveniles/ g root
Plant extracts (P)	***	**	***	***	***	***
Application (A)	***	*	**	*	***	***
Interaction						
P×A	Ns	Ns	***	***	***	Ns

For statistical details, see Table I.

The effect of the protective and curative application of *M. oleifera* extracts on the reproduction and growth of *C. jambhiri* was compared. Protective treatment by methanolic *M. oleifera* extracts was most effective to decrease the reproduction rate of *T. semipenetrans* i.e., juveniles/100mL soil, females/g root, and juveniles/g root followed by

ethanolic and aqueous extracts (Table VII). Protective application of methanolic extract also showed maximum plant growth. Root length, plant height, and the number of leaves were maximum in plants subjected to the protective treatment of methanolic extract followed by ethanolic and aqueous extracts (Table VIII). Protective application of all plant extracts was more effective to decrease the reproduction of *T. semipenetrans* and increasing the growth of plants. An inverse relation between the reproduction of nematodes and plant growth was observed. It can be concluded that *M. oleifera* extracts had the potential to decrease the development and reproduction of *T. semipenetrans,* thereby boosting plant growth.

Table VII. Effect of protective vs curative application of *M. oleifera* extracts on reproductive parameters of *T. semipenetrans.*

Treatment	Juveniles/ 100mL soil	Females/g root	Juveniles/g root
Protective application			
Methanolic extract	335.7e	33.0h	410.0f
Ethanolic extract	548.7d	50.0g	508.0ef
Aqueous extract	643.0cd	59.0f	603.3de
Control	1880.7a	350.3b	1633.3b
Curative application			
Methanolic extract	545.7d	101.3e	635.0de
Ethanolic extract	669.3cd	136.7d	766.7cd
Aqueous extract	769.7c	172.3c	905.3c
Control	1677.7b	587.7a	1950a

For statistical details, see Table II.

Table VIII. Effect of protective vs curative application of *M. oleifera* extracts on growth parameters of rough lemon *C. jambhiri.*

Treatment	Plant height (cm)	Root length (cm)	No. of leaves
Protective application			
Methanolic extract	17.0a	3.00abc	24.0a
Ethanolic extract	14.7b	3.66a	20.0b
Aqueous extract	11.3cd	2.50abc	16.6c
Control	9.70de	2.66abc	14.0c
Curative application			
Methanolic extract	15.0b	3.46ab	15.0c
Ethanolic extract	11.3c	2.56abc	15.3c
Aqueous extract	10.0de	2.06bc	11.0d
Control	8.50e	1.70c	9.00d

For statistical details, see Table II.

DISCUSSION

Plant-parasitic nematodes are an economically important group of soil-borne pathogens that are attempted to control though resistant germplasm, cultural practices, nematicides, and botanical extracts. Nematodes are a vast group of multicellular animals on earth representing 2/3rd of the total fauna (Khan, 1993). Hence, the magnitude of damage they cause to crops is immense. *T. semipenetrans* is a theatening pest of citrus around the globe. The root injuries inflicted by citrus nematodes can increase the risk of damage by other pathogens. Although, nematicides are effective to control nematodes, however, most pesticides have been banned due to the high risk to human health and the environment (Veremis and Roberts, 1996). The biological control of nematodes using antagonistic microbes and botanical extracts has become one of the most reliable alternatives to nematicides (Stirling, 1991).

The *in vitro* and *in vivo* application of *M. oleifera* extracts caused significant larval mortality of *T. semipenetrans*. The results of the present investigation showed that methanolic extract of *M. oleifera* inhibited the development of nematodes under field conditions, which is in line with the previous findings (Hafeez *et al*., 2000; Sharon *et al*., 2001; Claudius-Cole *et al*., 2010). *M. oleifera* contains toxic compounds such as saponin, tannins, alkaloids, steroids, and reducing sugars. The mortality of nematodes is due to the antimicrobial effect of these compounds. These basic phytochemicals confer a bio-nematicidal effect (Fatoki and Fawole, 2000; Izuogu and Oyedunmade, 2009). Some of these phytochemicals such as tannins and saponins are reported to possess a nematicidal effect disrupting biological membranes, thereby, facilitating the penetration of toxic compounds (Agrios, 2005). *M. oleifera* extract has been used against several plant-parasitic nematodes and they didn't show any phytotoxic effect which indicates that they are safe for use (Claudius-Cole *et al*., 2010; Izuogo *et al*., 2013; Sowley *et al*., 2014).

M. oleifera extracts reduced the penetration and reproduction of nematodes. Therefore, it has been concluded that methanolic extract of *M. oleifera* proved to be very effective in suppressing the development and reproduction of *T. semipenetrans* under field conditions. In the present investigation not only, the reproduction of *T. semipenetrans* was reduced but plant growth was also enhanced. Ahmad *et al*. (2004) reported that aqueous extracts of *Azadirachta indica, Datura alba,* and *Calotropis procera* caused larval mortality and reduced the multiplication and reproduction of *T. semipenetrans* on *C. jambhiri*. The protective treatment of *M. oleifera* extracts was more effective than curative. The protective effect may be due to the presence of inhibitory chemical compounds in *M. oleifera*

extracts. Shawky and Al-Ghonaimy (2015) support the present investigation, where, they stated the effect of 3 plant extracts *Origanum majorana*, *Tagetes erecta,* and *Eucalyptus globules* on *T. semipenetrans* and reported a reduced population of nematode and enhanced growth of the plants. The lowest number of females and J2 was observed in the protective application of *M. oleifera* extract. The growth parameters were also enhanced by the application of *M. oleifera*. Devi (2002) evaluated the effect of *Ocimum sanctum*, *Azadirachta indica, Myristica malabarica, M. oleifera, Mangifera indica*, and *Acacia Arabica* on mung bean, cowpea, and urdbean against nematode infection and observed that they not only reduced nematode population but also enhanced plant yield. The growth-promoting effect of plant extracts has been also reported previously by Awan *et al*. (1992) and Murslain *et al*. (2014), who, observed decreased population of nematodes and better growth of the plant. The application of *M. oleifera* extracts decreased the reproduction rate of nematodes and promoted the growth of plants. *T. semipenetrans* affects the root system directly, thereby, hindering the uptake of minerals and water that ultimately results in weaker plants. The decline in nematode reproduction decreased the disease attack that aided in promoting plant growth.

It can be concluded that the use of *M. oleifera* extract can be helpful to control *T. semipenetrans*. The use of botanical extracts will probably be an economical and effective approach. The active components of *M. oleifera* extract if isolated in pure form and the bulk amount will form a base for the development of bionematicides. Hence, the future direction of the study will be to test the efficacy of *M. oleifera* extracts on a large scale and study the active ingredients involved in the inhibitory effect.

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Generative AI and AI-assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statemnent of conflict of interest

The authors have declared no conflict of interest.

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