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THE EFFECT OF PLANT EXTRACTS OF SAGE (*SALVIA OFFICINALIS)* AND THYME (*THYMUS VULGARIS*) IN THE CAUSE OF DISEASE COMPLICATING THE ROOTS OF NEMATODES *MELOIDOGYNE INCOGNITA* ON THE TOMATO IN IRAQ

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Abstract

The parasitic nematodes are a group of pathogens that have been destroyed for agricultural production worldwide and their control is arduous and stressful, as well as pollution by bioaccumulation of pesticide residues and the emergence of resistance. The study included the plant extracts, including the extract of ethyl acetate and ethyl alcohol for the sage (*Salvia* sp.) and thyme (*Thymus vulgaris*) plants and their powder in three concentrations of 1000, 2000 and 4000 ppm in laboratory and in field of 2000 and 4000 concentration as alternative chemicals to control of nematodes. The Morphological nematode was diagnosed and found nematode eggs length between 94-98 µm, width 41-44 µm, and length of juvenile second stage (J2) between 351-399 µm and female length 618-680 µm and width 439-460 µm. The molecular nematode diagnoses based on rDNA sequences were shown molecular weight of 760 bp belonging to the *M. incoginta* nematode and the Iraqi nematode isolates were recorded in Gen Bank under accession numbers: MH509982, MH509983, MH509984, MH509985, MH509986 and MH509987. Laboratory experiment, the corrected rate of inhibition of nematode eggs hatching was significantly different from 5.8 to 34.47% after 24 hours of treatment with all extracts, while the ratio ranged from 10.73 to 48.10% after 48 hours and 72 hours after the incubation rate ranged from 17.02 to 72.34%. The results ofthe effect of the plant extracts in a preventive method showed that all treatments for thyme and sage were significant in reducing the disease severity 0.41-0.65 while in the control treatment 3.61. The disease index was 40.1-60.5% compared with control treatment 95.6%. The curative method results were showed diseases severity 0.61-0.58 while in the control treatment was 2.83 and the diseases index was between 40.2-80.5% compared with control treatment 90.7%.

Keywords: Sage and thyme extracts, *Meloidogyne incognita*, rDNA, tomato.

Introduction

Plant nematodes are major, important and dangerous pests in global plant production due to economic losses. Nematodes are considered to be the world's leading crop-causing pathogens and cause losses of up to $ 173 billion (Eilling, 2013). The nematodes are the most important nematode species Responsible for significant losses in the world's agricultural production systems (Moens *et al*., 2009). To reduce these losses, researchers discover new alternatives methods to control. One of these alternatives using plant extracts that have spread and expanded than other methods because it was impact on human, animal and natural enemies (Schwan- Estrada and Stargarlin, 2005). Sage (*Salvia* sp.) and thyme (*Thymus vulgaris*) plants belong to the lamiaceae family were contain on thyme oil, carvacrol and 30 chemical compounds (Shazia and Muzafar, 2011). The nematode eggs and juveniles were exposed to concentrations as 0.5%, 1%, 2.5%, 5% and 10% during 24, 48 and 72 hrs. As a result, bead tree (*Melia azedarach*) 10% (v/v) concentration significantly reduced the egg hatchability (97%) and elderberry (*Sambucus nigra*) (92.9%). The results of this investigation show this plants contain nematicidal compounds (Akyazi, 2014). Aqueous extracts of some plants were screened for egg hatchability and nematicidal activity against J2 of *M. incognita* in the laboratory. The nematode egg and juveniles were exposed 12, 24 and 48 h in four concentrations of plant extracts. The plant extracts of leaves of six plants exhibited highly promising mortality of 72 –99 % after 48 h of exposure (Asif *et al*., 2014). The Aydinli and Mennan,(2014) referred to the effect of cold extract plant of *Viscum album* had the lowest tomato root galling by followed hot plant extract of *Myrtus communis* and *Capsella bursa-pastoris* against *Meloidogyne arenaria*. The thyme leaves powders used to control of the citrus slow decline nematode in concentration of 1%, 2% and 4%, their resulted were showed nematodes destruction of 24.68%, 31.58% and 41.25% respectively (Nahid *et al*., 2016). The efficacy of some essential oils on the number of nematode egg masses and root gall formation on crop roots were found well enough at rate doses of 3% and 5% on *M. incognita* infected tomato plants grown in pots (Ozdemir and Gozel, 2018).The objective of this study was evaluated

of the sage (*Salvia* sp.) and thyme (*Thymus vulgaris*) plants extracts for effects on *M. incognita* on tomato in Iraq.

Material and Methods

Plant Extracts

The sage (*Salvia* sp.) and thyme (*Thymus vulgaris*) leaves plants dry used in this study were purchased from domestic marked. Extracts were prepared two different types as ethyl acetate and ethyl alcohol solvents. To prepare the extract, 100 grams of plant material was soaked in 200 ml of solvents for a 24 hour. The plant extracts were then squeezed through a cotton cloth and each extract was filtered through a Whatman filter paper (No. 1) stored at 40°C for overnight to evaporate solvents. The plantextracts were collect and kept at -20°C in dark bottle until used (Harborne, 1973).

Soil Samples Collection

The soil samples were collected during the spring season in 2018 from 5 different agricultural areas in Diyala Governorate (Hebbh, Hashimiyat, Jadida al-Shat, Khallas and Bani-Saad) and one soil samples from the agricultural research center of the College of Agricultural Engineering Sciences - Baghdad University in Jadiriyah, Baghdad - Iraq. The soil samples were distributed on plastic pots and planted with two seedlings from super-virgin tomato and according to Shuetleff and Averre (2000).

Nematode Culture

The culture of *M. incognita* eggs were extracted from infected tomato roots with 5% commercial bleach solution (1% chlorine free). Eggs released from the roots were collected on a 25 mesh sieve and transferred into distilled water. Second juveniles were obtained by placing egg masses into hatching vessel that were put in petri dishes adding 3 ml water. After 1 day, second juveniles were collected from water (Hussey and barker, 1973).

Nematodes Identification

Nematodes were morphological studies identified using light microscopy. Measurement dimensions for 20 individual from nematode eggs, juvenile second stage (J2) and female nematodes (Mekete *et al*., 2012).

Nematodes DNA Extraction

Individual gall from tomato infection were used for DNA extraction using genomic DNA Mini Kit (plant) for purifying total DNA from tomato root gall, dependent on Geneaid protocol.

Molecular Identification

The rDNA primer were used to carry out PCR assays to specifically identify *Meloidogyne* species. Forward primers 18s (5' TGA TTA CGT CCC TGC CTT 3') and Reveres primer 26s (5' TTT CAC TCG CCG TTA CTA AGG3') were used to amplify the region of the ribosomal DNA (rDNA) (Vrajn *et al*., 1992). PCR conditions included initial denaturing at 95 ^{0}C for 5 min., followed by 35 cycles of 95 ^{0}C for 50 sec., annealing 55 ^{0}C for 50 sec., and 72 ^{0}C for 1.5 min; followed by a final extension at 72 ^{0}C for 10 min. Amplification PCR products were sequenced by Sanger sequencing method in Bio-gene corporation (south Korea). The nematode sequences were deposited in Gen Bank and aligned using Cluster W (Landa *et al*., 2008). A phylogenetic analysis was performed with Mega 7 (Kumar *et al*., 2016).

Plant extract effect on egg hatching in Laboratory

Nine ml of each plant extract was added to each petri dish containing 250 eggs in a 1 ml of distilled water. Controls were established by adding 9 ml distilled water to a 1 ml of nematode suspension. The each treatment was replicated three times. The petri dishes were kept at 25°C and the total number of hatched juvenile was counted after 24, 48 and 72 hour. Results were expressed as the percent mortality = eggs hatch/total number of eggs *100 according for three periods.

Effects of plant extracts in pot experiment

Twenty one days old tomato seedlings cv. Super Regina were transplanted into plastic pots containing 250 cm^3 of autoclaved soil. Experiment was set up to see the effect of plant extract in preventive and curative methods on nematode. The experiment pots were inoculated by 1 ml of a suspension containing 1000 eggs. Twenty milliliter of extract was poured near roots as soil drench before and after 7 days from adding nematode inoculation. Distilled water and Carfacrol (Nematicide) were used as controls treatment. Experiment was designed random in a completely randomized block design with five replicates in greenhouse at 28°C. Sixty days after inoculations, plants were uprooted and disease severity and disease index were measured according for root gall index.

Results and Discussion

Morphological characterization of nematodes eggs, second-stage and juveniles of the populationof *Meloidogyne* sp. under study were compared with typical descriptions of *M. incognita*. Adult females were pyriform and lacking a terminal protuberance in the posterior area of the body (Fig.1 g-k). The female body

lengths ranged between 618 – 680 µm. Second-stage juveniles (Fig. 1e) averaged 351– 399 µm in length and 12 – 15 µm in width. Nematode eggs length between 94-98 µm with width between 41-44 µm andwere consistent with typical *M. incognita* in agreement with Calderón-Urrea *et al*. (2016), who described *M. incognita*.

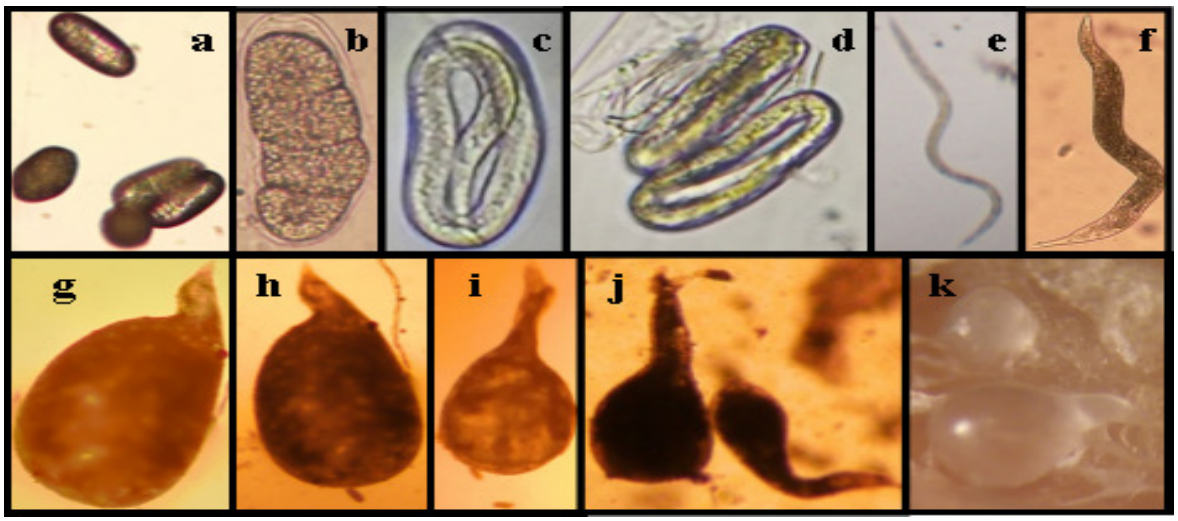

Fig. 1: Micrographs of eggs (a-c), second-stages juveniles (d-f) of *Meloidogyne incognita* from Iraq, females (g-k).

Phylogenetic analysis: the DNA sequences of the internal transcribed spacer (ITS) of rDNA genes for *M. incognita* have beenuseful to identify specie of nematodes (Vrain *et al.,* 1992). An approximately 760bp DNA fragment of the nematode population under study. Sequences of the rDNA were aligned with others Meloidogyne species obtained from Gen Bank (https;//www.nebi.nlm.nih.gov). The Maximum Likelihood tree (Fig. 2) was highly resolved and showed that the population of *M. incognita* was studied formed a clade together with sequences from other *M. incognita* from Gen Bank with high bootstrap support. Here, sequencing the rDNA region of the population was isolated aided our diagnosis of *M. incognita*. The population under study belonged to *M. incognita*.

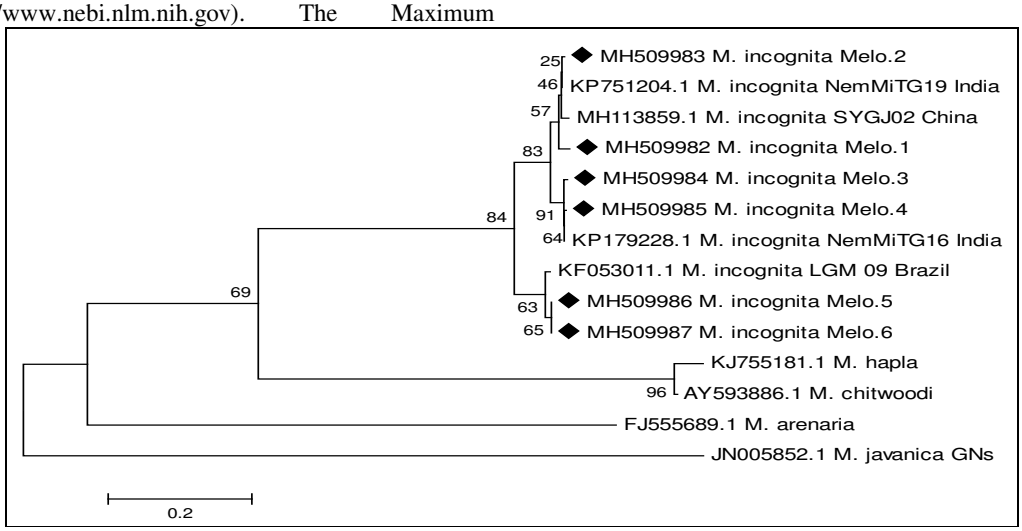

Fig. 2: Phylogenetic tree of the genus *Meloidogyne incognita* based on the ITS of rRNA, bootstrap support values ♦ = 50%. Gen Bank numbers of Iraqi nematode.

Plant extract effect on egg hatching in Laboratory

All plant extracts tested in this study were effected egg hatching and of *M. incognita* in petri experiments (Table 1). The result shows the effect of plant extracts on accumulative egg hatches of *M. incognita*. Laboratory experiment, the corrected rate of inhibition of nematode eggs hatching was significantly different from 5.8 to 34.47% after 24 hours of treatment with all extracts, while the ratio ranged from 10.73 to 48.10% after 48 hours and 72 hours after the incubation rate ranged from 17.02 to 72.34%.

When the comparing the efficacy of these extract preparation methods, present study showed that effective in petri experiments. These results are consistent with the result presented by Akyazi (2014), referred to the nematode eggs and juveniles were exposed to concentrations as 0.5%, 1%, 2.5%, 5% and 10% during 24, 48 and 72 hrs. As a result, bead tree (*Melia azedarach*) 10% (v/v) concentration significantly reduced the egg hatchability (97%) and elderberry (*Sambucus nigra*) (92.9%). The results of this investigation show this plants contain nematicidal compounds.

Table 1 : Effect of plant extracts on egg hatching of *M. incognita.*

No.	Plant extract	Concentration/ ppm	Eggs hatch (%)*		
			24 h	48 h	72 h
1	Sage alcohol Ethyl acetate	1000	7.48*	21.11	25.53
2	Sage alcohol Ethyl acetate	2000	13.99	29.41	38.29
3	Sage alcohol Ethyl acetate	4000	22.18	48.10	68.08
4	Sage Ethyl alcohol	1000	7.48	10.73	17.02
5	Sage Ethyl alcohol	2000	13.75	19.03	25.53
6	Sage Ethyl alcohol	4000	16.04	33.56	44.64
7	Thyme Ethyl alcohol acetate	1000	7.48	15.92	25.53
8	Thyme Ethyl alcohol acetate	2000	13.99	27.54	34.04
9	Thyme Ethyl alcohol acetate	4000	14.96	33.56	72.34
10	Thyme Ethyl alcohol	1000	5.80	14.88	21.27
11	Thyme Ethyl alcohol	2000	7.48	23.18	29.78
12	Thyme Ethyl alcohol	4000	14.96	39.79	46.80
13	Sage powder	1000	11.94	19.03	21.27
14	Sage powder	2000	20.14	31.49	38.29
15	Sage powder	4000	34.47	48.10	51.06
16	Thyme powder	1000	11.94	23.18	29.78
17	Thyme powder	2000	20.14	31.49	40.72
18	Thyme powder	4000	28.33	43.74	46.80
19	Carfacrol pesticide	10 %	100	100	100
L.S.D. 0.05			02,194	0.1482	0.1342

* Percentage egg hatch in relation to current hatch in control after 24, 48 and 72 days.
* A very number in the table equal three replicates.

Effects of plant extracts in pot experiment

The application of plant extracts to inoculated plant had a significant reduction of disease severity and disease index compared with control treatment. The results of the effect of the plant extracts in a preventive method showed that all treatments for thyme and sage were significant in reducing the disease severity reached 0.41-0.65 while in the control treatment 3.61. The disease index was reached 40.1-60.5% compared with control treatment 95.6% (Table 2).

Results of pot experiment of the curative method results were showed diseases severity for plant extracts reached 0.61-0.58 while in the control treatment was 2.83. The diseases index was reached between 40.2 - 80.5% compared with control treatment 90.7% (Table 3).Similar results found by Oka *et al.* (2000) which used that different species of the Mentha plant (*M. rotundifolia*) exhibit excellent effective activity in vitro and pot experiments. The nematode eggs and juveniles were exposed to concentrations as 0.5%, 1%, 2.5%, 5% and 10% during 24, 48 and 72 hrs. As a result, bead tree (*Melia azedarach*) 10% (v/v) concentration significantly reduced the egg hatchability (97%) and elderberry (*Sambucus nigra*) (92.9%). The results of this investigation show this plants contain nematicidal compounds (Akyazi, 2014). The efficacy of some essential oils on the number of nematode egg masses and root gall formation on crop roots were found well enough at rate doses of 3% and 5% on *M. incognita* infected tomato plants grown in pots (Ozdemir and Gozel, 2018).

Table 2 : Effect of plant extracts on tomato inoculating with *M. incognita* (in preventive method).

No.	Plant extract	Concentration/ ppm	Preventive method	
			Disease severity	Disease index
1	Sage alcohol Ethyl acetate	2000*	0.42	% 40.2
2	Sage alcohol Ethyl acetate	4000	0.43	% 40.3
3	Sage Ethyl alcohol	2000	0.65	% 60.5
4	Sage Ethyl alcohol	4000	0.41	% 40.1
5	Thyme Ethyl alcohol acetate	2000	0.62	% 60.2
6	Thyme Ethyl alcohol acetate	4000	0.45	% 40.5
7	Thyme Ethyl alcohol	2000	0.44	% 40.4
8	Thyme Ethyl alcohol	4000	0.46	% 40.6
9	Sage powder	2000	0.63	% 60.3
10	Sage powder	4000	0.42	% 40.2
11	Thyme powder	2000	0.61	% 40.1
12	Thyme powder	4000	0.43	% 40.3
13	Carfacrol pesticide	10 %	0.25	% 20.5
14	Control treatment		3.61	% 95.6
L.S.D. 0.05			0.1762	10.138

* A very number in the table equal five replicates.

Table 2 : Effect of plant extracts on tomato inoculating with *M. incognita* (in curative method).

No.	Plant extract	Concentration / ppm	Curative method	
			Disease severity	Disease index
1	Sage alcohol Ethyl acetate	2000*	0.81	% 80.1
2	Sage alcohol Ethyl acetate	4000	0.63	% 60.3
3	Sage Ethyl alcohol	2000	0.85	% 80.5
4	Sage Ethyl alcohol	4000	0.62	% 60.2
5	Thyme Ethyl alcohol acetate	2000	0.84	% 80.4
6	Thyme Ethyl alcohol acetate	4000	0.66	% 60.6
7	Thyme Ethyl alcohol	2000	0.69	% 60.9
8	Thyme Ethyl alcohol	4000	0.61	% 60.1
9	Sage powder	2000	0.83	% 80.3
10	Sage powder	4000	0.62	% 60.2
11	Thyme powder	2000	0.84	% 40.2
12	Thyme powder	4000	0.85	% 40.5
13	Carfacrol pesticide	10 %	0.45	% 40.5
14	Control treatment		2.83	% 90.7
L.S.D. 0.05			0.1586	10.466

* A very number in the table equal five replicates.

Root-knot nematodes are causal serious disease of tomato and these pathogens are appealing a more significant damage on tomato plants in different regions.

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