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Evaluation of Metabolites of *Myrothecium verrucaria* as Biological Nematicide against Root-knot Nematode, *Meloidogyne incognita* in Vitro and in Vivo on Sugar Beet Plants

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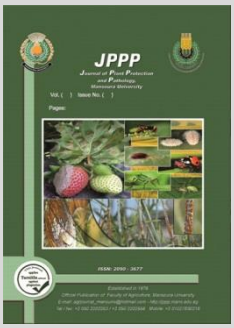

ABSTRACT

Nematicidal activity of heat-sterilizing filtrate culture of *Myrothecium verrucaria* was evaluated against *Meloidogyne incognita* in *Vitro* and the metabolites formulated as granules of *M. verrucaria* were prepared and tested on sugar beet plants infested with *M. incognita* comparison with DiTera™ and Rugby 10%G, under greenhouse conditions. Laboratory results showed that sterilized culture filtrate of the fungus *M. verrucaria* either by syringe or by autoclaving significantly reduced the percentages of egg hatching and increased the percentage of 2nd stage juveniles (J_2) mortality of *M. incognita*. The most effective reductions of hatching were obtained at 50% concentration with the sterilized filtrate by autoclaving (94.23%), while the most effective reached 96.70% increasing in the percentage of mortality of J_2 after 3 days from treating by 50% concentration in case the sterilized filtrate by syringe. Results of greenhouse experiment showed that, all treatments by the prepared fungal formulae of *M. verrucaria* or DiTera with different rats significantly reduced the number of galls on sugar beet roots and final nematode population (J_2) in soil compared with control (nematode only). The higher rate (0.2 g /pot) was achieved the highest reduction in root-galling (86%) with DiTera treatment and the highest reduction in (J_2) with fungal formulae treatment (92.2%). Improvement in plant growth parameters were noticed by treatments. Analysis by GC-MS of the fungus metabolites was identified compounds to have antimicrobial and nematicidal activities. The prepared formulae from the Egyptian isolate of *M. verrucaria* can be used as safety bio-nematicide against *M. incognita* on sugar beet.

Keywords: *Myrothecium verrucaria*, Bio-nematicide, *Meloidogyne incognita*, sugar beet

INTRODUCTION

Meloidogyne species constitute the major nematode problems in developing countries. Root-knot nematodes are ranked at the top among the five major plant pathogens and the first among the ten most important genera of plant parasitic nematodes in the world (Mukhtar *et al*., 2017). They have wide geographic distribution, large host range and high destructive potential. In Egypt, *Meloidogyne* spp. is the most frequently encountered nematode genera, with their frequency of occurrence being greater than 50% (Ibrahim, 1994). In northern Egypt the root- knot nematodes, *Meloidogyne incognita* and *M. javanica* were very common on the most crops (Ibrahim and Handoo, 2016).

Sugar beet (*Beta vulgaris*), is an important arable crop, that traditionally used for sugar extraction, and recently, for biofuel production. In Egypt, sugar beet is cultivated in 219087 ha. with production of about 11222720 tones (FAO STATE, 2018). The most serious problem against sugar beet extension in new areas is root-knot nematode. *M. incognita* and *M. javanica* were reported as major nematode pests of sugar beet in Egypt, (Ibrahim, 1982; Oteifa and El-Gindi, 1982; Abd El-Massih, 1985; Ismail *et al*., 1996 and Maareg *et al*., 1998).

Various nematicides have been so far used to control root-knot nematode are not only expensive but also hazardous to human and soil health (Kumari *et al*., 2020). To address this issue, eco-friendly control measures, such as microbial nematicides, are being developed (Nguyen *et al*., 2018).

The fungus *Myrothecium verrucaria* produces a biologically active metabolite or metabolites in various fermentation media. Both the fungus and its metabolites have nematocidal activity and, thus, prevent plant damage from nematodes and control the growth of nematodes (Devidas and Crovetti, 1991). *M. verrucaria* or its culture filtrate were highly effective against hatched juveniles and increase larval mortality of the root-knot nematode, *Meloidogyne* spp. (Hagag, 2000 and 2009; Kepenekci *et al*., 2017 and Nguyen *et al*., 2018). The fungal and its metabolites can be used to control nematodes for a variety of agricultural applications on many different plants and fruits. *Myrothecium* species exhibited strong insecticidal activities against nematodes, promising antimicrobial activities, and are involved in many biotechnological applications due to secondary metabolites produced by them (Elkhateeb and Daba, 2019).

DiTera, a commercial formulation of *M. verrucaria* (valent U.S.A) which is known to be produced commercially as new biological nematicides. It can be used to control nematodes for a variety of agricultural applications on many different plants and fruits. (Warrier *et al*., 1999). The product and its formulations have been

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registered as a microbial nematicide in several countries. Field and greenhouse evaluations of DiTera on turf, bananas, fruit and vegetable crops indicate a significant reduction in population of the major nematode pests affecting those crops, including root-knot, cyst, sting and burrowing nematodes, during the critical stages of plant growth. (Westerdahl *et al.,* 2004; Ami and Sipes, 2008; Khalil *et al*., 2010; Shawky *et al*., 2010 and Lewis 2017).

As reported in our previous studies (Hagag, 2000 and 2009), the Egyptian fungal isolate, *M. verrucaria* was proved to be antagonistic against *Meloidogyne* spp..

The present study aimed to evaluating the nematicidal activity of the sterilized culture filtrate of *M. verrucaria* either by syringe or by autoclaving against root-knot nematode, *M. incognita* in the laboratory to prove that the activity of metabolites of *M. verrucaria* are substantially unaffected by changes of temperature to preparation of the fungal metabolites by modification method to formulate bio product safe and easy for preparation to studying its effects on *M. incognita* in sugar beet plants under greenhouse conditions.

MATERIALS AND METHODS

Source and inoculum preparation of the root-knot nematode (*Meloidogyne incognita*):

Individual egg-masses of distinct root-knot nematode (*M. incogneta*) were collected from diseased sugar beet roots from Sakha agricultural research farm, kafrelsheikh governorate by the aid of a special needle .A stock culture of the 2nd stage juvenile J_2 were obtained from the collected mature egg-masses after immersion in sterilized water for 7-10 days. The obtained health newly 2nd stage juveniles were reared on tomato seedlings planted (Super streen B) in pots filled with sterilized soil under greenhouse conditions. The tomato seedlings were maintained in greenhouse at the Department of Plant Pathology, Sakha Agricultural Research Station for more than 45 days to prepare eggs to obtain nematode inoculum. The re-extracted nematodes from diseased tomato plants were identified by perineal patterns according to Barker (1985). Identification of *M. inognita* was confirmed by polymerase chain reaction (PCR) using CTAB protocol according to Allen *et al.*, (2006) at Central Laboratory of Bioinformatics, Plant Pathology Research Institute, A.R.C. Egypt. Inoculum of *M. incogneta* was prepared by NaOCl solution (Hussey and Barker, 1973) and was adjusted to deliver a suspension of 5000 eggs per plant.

The fungal isolate:

In our previous study the Egyptian fungal isolate, *M. verrucaria* was isolated from infected 2nd stage juveniles of *Meloidogyne* sp. and was identified by Prof. W. Gams, Central Bureau Voor Schimmelcultures, Baan, Netherlands (Hagag, 2000).

Activity of heat-sterilizing filtrate culture of *Myrothecium verrucaria* against *M. incognita* in the laboratory:

Fungal culture in medium broth (50 g sucrose, 1.3 g K_2HPO_4, 1 g KH_2PO_4, 0.5 g $(NH_4)2So_4$, and 0.5 g $MgSo_4.7H_2O$ in each liter of distilled water) was grown for 30 days in 500 ml. flasks at 27 °C. The fungal mycelium was removed and the liquid medium was filtrate through Whatman No. 1 filter paper then, was centrifuged (9000 r pm for 30 min.). The resulted supernatant was filtrated through syringe filter 0.2 µm (Whatman, Clifton, NJ, USA) or autoclaved at 121°C. for 20 minutes. The sterilized filtrate, either by syringe or by autoclaving, has been diluted with sterilized distilled water to 1, 5, 10, 25, 50% concentrations of the standard filtrate. Ten ml. of each concentration was pipetted in 25 ml vials which containing 1 ml of eggs suspension or the second juveniles (approximately, 1000 eggs or J_2) of *M. incognita*. Control treatment was sterilized distilled water. Each treatment was replicated three times. After incubation for 24, 48 and 72 hr. at 27°C., the numbers of live and dead larvae were counted and the percentages of larvae mortality were calculated. Fourteen days after incubation, data were recorded for hatching percentage using the research microscope.

Production and formulation of antagonistic metabolites from *M. verrucaria*:

The fungus *M. verrucaria* was grown on PDA medium. After three weeks of growth at 25 °C., spores were transferred into 100 ml of sterilized liquid seed medium (PD) in 500ml flasks. Flasks were incubated with shaking at 27°C for three days to obtain seed culture. Fermentation medium [200 ml in 500 ml flask, composed of 2g glucose, 15.0g soluble starch, 2.0g yeast extract, 0.3g hydrated magnesium sulfate, 1.0g calcium carbonate and 5.0g pepton in 1liter of water at an adjusted PH 7.0 prior to autoclaving], was inoculated with 1-2 % v of seed culture. Flasks were incubated at 25°C for 5 days with agitation at 200 r.p.m. (Devidas and Crovetti, 1991). After cultivation, the culture thus obtained is autoclaved at 121°C., for 20 minutes. The obtained whole culture which contains sterilized metabolites and the biomass (Fig. 1) was frozen and lyophilized. To preparation the formulae as dispersible granules of *M. verrucaria*, the prepared lyophilized culture was stirred with 5% W/W kaolin, 5% W/W synthetic hydrated silicon oxide powder and some drops of Arabic gum then, to the mixture is added an appropriate amount of water, granulated and air-dried to obtain dispersible granules (Modified method according to Hagag, 2009).

Figure 1. Culture of fungus *Myrotheciu verrucaria* in fermentation medium.

Effect of *M. verrucaria* formulae against *M. incognita* on sugar beet plants under greenhouse conditions

The experiment:

In 2017/2018 season, sugar beet seeds cv. Glorius were sown in 26 cm. diameter pots filled with about 8 kg a mixture of clay and sand soil (2:1 v:v). At fourth the leaf stage, the plants were thinned into two seedlings per pot. Then, each plant was inoculated with approximately 5000 eggs of *M. incognita.* The inoculum was put inside three

holes in the soil, around the base of stems, and then the pots were irrigated. The prepared granules of *M. verrucaria* were added to the infested soil with root-knot nematode at three different rates i.e. 0.05, 0.1 or 0.2 g /pot for 4 times with 15 days intervals. DiTera was added with the same rates. DiTera was kindly delivered from Dr. Ashraf Khalil, head of Plant Pathology Research Institute, Agricultural Research Center, Egypt. Each treatment was represented by four pots. Check treatment and treatment with comparable nematicide Rugby 10%G (Cadusafos) were also included. Rugby was broadcasted and incorporated in soil at the rate of 0.17 g/pot (24 kg/fed.) as recommended. All pots were arranged in the greenhouse in a completely randomized design, and kept at 20±5°C. at the Department of Plant Pathology, Sakha Agricultural Research Station.

After 5 months, plants were up rooted and number of galls, gall index (GI) and final nematode population (J_2) in 250 cm3 soil were determined. Gall index was determined according to Sharma *et al*. (1994) as follows: 1 = no galls, 2 = 1 to 5 galls, 3 = 6 to 10 galls, 4 = 11 to 20 galls, 5 = 21 to 30 galls, 6=31 to 50 galls, 7 = 51 to 70 galls, 8 = 71 to 100 galls, and 9 = >100 galls per root system. Also data on plant growth was measured, root length, fresh weigh and dry weigh of root and shoots, number of leaves. Percentage of total soluble solids (T.S.S. %) was measured in the fresh root using hand refractometer (Carruthers and Old field 1961).

GC-MS analysis:

Metabolites identification by gas chromatography massspectrometry (GC-MS) was done at Central Laboratory of Bioinformatics, Plant Pathology Research Institute, A.R.C, Egypt. For identification the fungus metabolites of *M. verrucaria* by GC-MS analysis, fungal isolate was grown on potato dextrose agar at 27 °C for 5 days. Three pieces (0.5–0.5 cm2) of mycelial agar plugs were inoculated into 1000mL Erlenmeyer flasks containing 500mL potato dextrose broth and incubated at room temperature for 28 days under stationary condition. The broth and mycelia were blended together and extracted with equal volumes of ethyl acetate and mycelia were removed by filtration through four layers of muslin cloth. The filtrates were evaporated using a rotary evaporator (Ruma *et al*., 2014). The obtained extracts of the fungus was frozen and lyophilized then, dissolved in methanol and used further for the assays. Metabolites identification by GC-MS was done to determine the volatile compounds in the fungus extracts. Dissolved extracts were diluted and injected into the gas chromatography (HP 6890N) equipped with mass detector (HP 5975) and a capillary column of fused silica HP 5-MS (30 m × 0.32 mm with film thickness 0.25 µm). The programmed oven temperature was gradually raised at a rate of 30°C per min until 230°C and then maintained for 20 min at 230°C. The detector was heated and injection port was 250°C. The carrier gas used was helium at 5 Psi pressure. The mass spectra were obtained by the parameters of ionization potential 70 eV, a temperature 250°C and mass range from 40–420. The identification of metabolites was based on their retention times and mass spectra compared to those compounds in the database library of NIST 98 L, Wiley 7n1 and Pest 1 by Chemstation Integrator computer software.

Statistical analysis:

Data collected were statistical analyzed using the completely randomized design. Analysis of variance (ANOVA) was performed by WASP-Web Agri. Stat. Package statistical analysis software. Treatment means were separated using Duncan's multiple range test (Duncan, 1955). All analyses were conducted at the significance value of $p \leq 0.05$.

RESULTS AND DISCUSSION

Results

Identification of root-knot nematode:

Illustration of typically symptoms of galls *M. incognita* on sugar beet roots from Sakha, Kafrelsheikh governorate was found (Fig. 2).

Fig. 2. Typically symptoms of sugar beet roots naturally infected with root-knot nematode, *Meloidogyne incognita*

Activity of heat-sterilizing filtrate culture of *M. verrucaria* against *M. incognita* in the laboratory:

Impact on percentage of egg hatching of *M. incognita:*

Data obtained in Table (1) indicated that, the culture filtrate of the fungus *M. verrucaria* significantly reduced the percentages of egg hatching of *M. incognita.*

Table 1. Impact of heat-sterilizing filtrate culture of *Myrothecium verrucaria* at different concentrations on percentage of egg hatching of *Meliodogyne incognita* under laboratory conditions

Methods of Sterilization	Concentration %	Egg hatching % after 14 days	Reduction %
Filtration	1	35.08b	53.32
	5	26.64c	64.55
	10	10.37d	86.20
	25	7.54def	89.96
	50	6.04ef	91.96
Heat-sterilization	1	30.06c	60.00
	5	9.68de	87.12
	10	6.53def	91.3
	25	4.53f	93.97
	50	4.33f	94.23
Control (untreated)		75.16 a	--------

Means followed by a common letter(s) are not significantly different at the 5% level by DMRT.

The most effective reduction of hatching was obtained at 50% concentration with the sterilized filtrate, either by syringe (91.96%) or by autoclaving (94.23%). However, there was no significant different among the last three concentration (10, 25 and 50%), and the last four concentrations (5, 10, 25, 50%) just in case, using the

sterilized filtrate by syringe or by autoclaving, respectively. In general, the percentage of egg hating decreased as the filtrate concentration increased.

Influence on mortality of the 2nd stage juveniles percentage of *M. incognita:*

Data presented in Table (2) and Fig (3) revealed that, all concentrations of *M. verrucaria* culture filtrate, except first concentration (1%) increased the percentage of inactive juveniles.

Table 2. Influence of heat-sterilizing filtrate culture of *Myrothecium verrucaria* at different concentrations on activity of the 2nd stage juveniles percentage of *Meloidogyne incognita* under laboratory conditions

Methods of Sterilization	Concentration %	mortality of 2nd stage juveniles after different exposure periods (days) %		
		1	2	3
Filtration	1	1.40a	1.89a	1.82a
	5	4.36a	21.27b	34.00c
	10	52.82b	58.13c	84.53e
	25	87.33cd	96.58d	97.33f
	50	87.57cd	97.78d	98.41f
Heat-sterilization	1	1.90a	1.90a	2.9a
	5	7.00 a	20.03b	24.43a
	10	51.50b	57.20c	65.44d
	25	73.25c	94.44d	96.49f
	50	90.19d	96.97d	96.70f
Control (untreated)		1.40a	1.50a	1.50a

Means followed by a common letter(s) are not significantly different at the 5% level by DMRT.

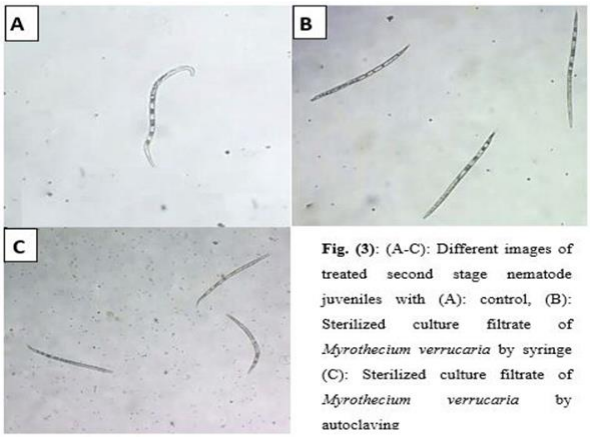

Fig. (3): (A-C): Different images of treated second stage nematode juveniles with (A): control, (B): Sterilized culture filtrate of *Myrothecium verrucaria* by syringe (C): Sterilized culture filtrate of *Myrothecium verrucaria* by autoclaving

The most effective reached 98.41% and 96.70% after 3 days from treating by 50% concentration just in case the sterilized filtrate by syringe and by autoclaving, respectively. Generally, the percentage of inactive juveniles increased as the filtrate concentration and the duration of exposure increased.

Effect of *M. verrucaria* formulae against *M. incognita* on sugar beet plants under greenhouse conditions

Disease parameters

All treatments by the prepared fungal formulae of *M. verrucaria* or DiTera with different rats significantly reduced the number of galls on sugar beet roots and final nematode population (J_2) in soil (Table 3). The highest reduction of root-galling (86.0%) was achieved when soil was treated with DiTera at the rate of 0.2 g/pot, followed by the fungal formulae of *M. verrucaria* at the rate of 0.2 g/pot (84.0%). The effect of these treatments in reducing root-galling was not significantly different from that achieved by Rugby 10%%G (84.8%). Also, Table (3) indicate that the highest reduction in nematode population (J_2) was achieved by soil treating with *M. verrucaria* formulae (92.2%), followed by DiTera (90.6%) at the rate of 0.2 g/pot. The effect of these treatment in reducing nematode population (J_2) was not significantly different from that achieved by the nematicide Rugby 10%G (92.3%).

Table 3. Effect of *M. verrucaria* formulae on some disease parameters of *Meloidogyne. incognita* on sugar beet under greenhouse conditions

Treatments	Concentration (g/pot)	Gall index	Number of galls /root system	Reduction %	Number of J_2/250 $cm^3$of soil	Reduction %
Formulae of *M. verrucaria*	0.05	9.0a	125.5b	49.8	45.0 b	64.8
	0.1	9.0a	150.0b	40.0	43.0b b	66.4
	0.2	5.0b	40.0c	84.0	10.0c	92.2
DiTera	0.05	9.0a	120.0b	52.0	40.0 b	68.7
	0.1	9.0a	126.0b	49.6	35.0b	72.7
	0.2	4.5b	35.0c	86.0	12.0c	90.6
Cadusafos 10% G	0.17	4.5b	38.0c	84.8	10.0c	92.3
Control (nematode only)		9.0a	250.0a	------	128.0a	------

Means followed by a common letter(s) are not significantly different at the 5% level by DMRT.

Some plant growth parameters and percentage of T.S.S of sugar beet plants

Data obtained in Table (4) revealed that all treatments significantly increased the fresh weight of plants compare with control 1 (with nematode), except soil treating with formulae of *M. verrucaria* (0.05g /pot) and didn't significantly differ from that achieved by control 2 (without nematode).

Table 4. Effect of *M. Verrucaria* formulae on some plant growth parameters and T.S.S% of sugar beet plants infested with *M. incognita* under greenhouse conditions.

Treatments	Concentration (g/pot)	Shoots weight (g/plant)		Root weight (g/plant)		Length of root (cm)	Number of leaves	T.S.S %
		Fresh	Dry	Fresh	Dry			
Formulae of *M. verrucaria*	0.05	46.74d	8.88cd	123.5d	17.6e	17.5 c	27.5 b	18.5d
	0.1	44.21d	9.55c	152.0bcd	23.5bc	21.0 b	28.5 b	22.0ab
	0.2	63.04a	11.63ab	183.5b	25.3ab	25.5 a	31.6 a	20.5bc
DiTera	0.05	50.16cd	9.66c	140.0cd	16.8ef	17.5 c	26.0 bc	16.5e
	0.1	43.13d	0.35bc	138.0cd	17.3e	24.5 a	25.5 bc	19.4cd
	0.2	65.33a	13.0a	175.0b	21.0cd	23.5 ab	31.5 a	21.0bc
Cadusafos 10% G	0.17	54.05bc	10.36bc	170.0bc	19.9de	21.0 b	23.5 c	21.3b
Control 1(with nematode)		35.44e	7.76d	133.5d	13.7f	17.5 c	17.0 d	15.3e
Control 2 (without nematode)		61.20ab	12.20a	226.5a	27.7a	21.0 b	23.5 c	23.5a

Means followed by a common letter(s) are not significantly different at the 5% level by DMRT.

Also, all treatments significantly increased the dry weight of shoots and roots and number of leaves compare with control 1 at all rates of treatments (Table 4), while only treatments at the higher rates significantly increased fresh weight of roots compare with control 1. All treatments significantly increased length of roots compare with control 1, except soil treating with formulae of *M. verrucaria* at 0.05g /pot. Also, data presented in Table (4) indicated that all treatments significantly increased the percent of T.S.S, except soil treating with Ditera at 0.5g /pot. Treatment with formulae of *M. verrucaria* at 0.2 g /pot was achieved the highest increased in fresh and dry of shoots and roots, length of roots, numbers leaves and percentage of T.S.S of roots (63.04 g, 11.63 g, 183.5 g, 25.3 g, 25.5 cm, 31.6, 20.5%, respectively).

Identified metabolites of *M. verrucaria* by GC-MS analysis:

Characterization of metabolites excreted by resulted in 13 compounds, according to the retention time and peak area percentage (Table 5 and Fig. 4).

Table 5. Metabolites from *Myrothecium verrucaria* determined by GC MS method.

Peak	RT(min.)	Area%	Name of compound
1	12.113	1.91	Carbonic acid, butyl Octadecyl ester
2	12.560	2.22	Ethanol , 2- (eicosyloxy)-
3	12.777	15.09	Thiocyanic acid, 2,4- dinitrophenyl ester
4	13.658	2.31	carbonic acid, butyl pentadecyl ester
5	14.276	6.86	1-Dodecanol, 2-hexyl-
6	15.083	2.07	Tetrapentacontane, 1,54-dibromo-1,54-Dibromotetrapentacontane
7	15.570	5.91	no matches found
8	15.713	1.59	14-.BETA.-H-PREGNA $$ 14-.BETA.-PREGNA $$ 14B-PREGNANE
9	15.879	12.03	no matches found
10	16.044	2.11	14-.BETA.-H-PREGNA $$ 14-.BETA.-PREGNA $$ 14B-PREGNANE
11	16.147	3.19	HAHNFETT
12	16.319	5.55	no matches found
13	16.554	2.90	1-Octadecene (CAS) $$ .alpha.-Octadecene $$ Octadecylene .alpha.-
14	16.874	0.28	14-.BETA.-H-PREGNA $$ 14-.BETA.-PREGNA $$ 14B-PREGNANE
15	17.200	5.48	Hexatriacontyl pentafluoropropionate
16	17.269	0.50	HAHNFETT
17	17.601	1.65	HAHNFETT
18	18.030	1.48	HAHNFETT
19	18.459	2.48	Dotriacontyl heptafluorobutyrate
20	24.015	3.80	3-Amino-7-nitro-1,2,4-benzotriazine 1-oxide
21	27.528	2.29	5,8-Epoxy-15-nor-labdane
22	27.763	0.67	Cyclotrisiloxane, hexamethyl-
23	27.946	-0.14	5,8-Epoxy-15-nor-labdane
24	28.198	4.99	5,8-Epoxy-15-nor-labdane
25	28.415	1.85	5,8-Epoxy-15-nor-labdane
26	28.593	1.00	5,8-Epoxy-15-nor-labdane
27	28.696	2.87	5,8-Epoxy-15-nor-labdane
28	28.747	1.17	5,8-Epoxy-15-nor-labdane
29	28.953	1.77	Cyclotrisiloxane, hexamethyl--
30	29.033	1.46	5,8-Epoxy-15-nor-labdane
31	29.188	1.22	5,8-Epoxy-15-nor-labdane
32	29.234	0.78	5,8-Epoxy-15-nor-labdane
33	29.400	0.99	5,8-Epoxy-15-nor-labdane
34	29.611	2.59	5,8-Epoxy-15-nor-labdane
35	29.691	1.68	5,8-Epoxy-15-nor-labdane

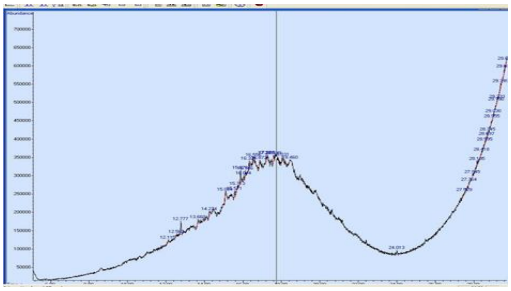

Fig. 4. Structural data of metabolites extracted by *Myrothecium verrucaria* GC-MS analysis.

The compounds were: 5, 8-Epoxy-15-nor-labdane (23.03%), Thiocyanic acid, 2,4- dinitrophenyl ester (15.09%), 1-Dodecanol, 2-hexyl- (6.86%), Hexatriacontyl pentafluoropropionate (5.48%), 14-.BETA.-H-PREGNA $$ 14-.BETA.-PREGNA $$ 14B-PREGNANE (3.98%), 3-Amino-7-nitro-1,2,4-benzotriazine 1-oxide (3.80%), 1-Octadecene (CAS) $$ .alpha.-Octadecene $$ Octadecylene .alpha.- (2.90%), Dotriacontyl heptafluorobutyrate (2.48%), carbonic acid, butyl pentadecyl ester (2.31%), Ethanol , 2- (eicosyloxy)- (2.22%), Tetrapentacontane, 1,54-dibromo-1,54-Dibromotetrapentacontane (2.07%), Carbonic acid, butyl Octadecyl ester (1.91%), Cyclotrisiloxane, hexamethyl- (1.77%), Cyclotrisiloxane, hexamethyl- (0.67%).

Discussion

Results of *in vitro* experiment showed that with the sterilized filtrate, either by syringe or by autoclaving at different concentrations significantly reduced the percentage of egg hatching and active juveniles of *M. incognita*. The percentage of inactive juveniles increases as the metabolites concentration increases and as the duration of the exposure increases. These results are in agreement with that reported by Devidas and Crovetti (1991), Hagag, (2009), Kepenekci *et al*., (2017) and Nguyen *et al*., (2018). Elkhateeb and Daba (2019) decided that, *Myrothecium* species exhibited strong insecticidal activities especially against insects and nematodes; promising antimicrobial activities and they are involved in many biotechnological applications due to secondary metabolites produced by them.

Laboratory results have drawn attention to there was no significant different between the sterilized filtrate, either by syringe or by autoclaving in their effects on reducing the percentages of egg hatching or increasing of inactive J_2 of *M. incognita*. These results indicate that the activity of metabolites of *M. verrucaria* are substantially unaffected by changes of temperature, this is decided by Devidas and Crovetti (1991). He also explained that the mycellil extract and filtrate as produced by fermentation of *M. verrucaria* and the fungus itself exhibit nematicidal activity against *M. incognita*. Hence, the heat-sterilized metabolite was used in preparation the formulae of *M. verrucaria* to formulate bio product safe and easy for preparation.

Data obtained in greenhouse showed that all treatments by the prepared fungal formulae of *M. verrucaria* or DiTera with different rats significantly reduced the number of galls on sugar beet roots and final nematode population (J_2) in soil and improved the growth of sugar beet plants. Comparing the performance of the tested *M. verrucaria* formulae to root-knot disease in sugar beet with DiTera, results revealed that the effect of these treatments in reducing root-galling and nematode population (J_2) in soil were do not differ

significantly between them. The general trend concluded from this experiment revealed that effect of the treatments increase by increasing the rates, they achieved significant high levels of protection against root-knot nematode at the higher rate (0.2 g/pot) which was not significantly different from that achieved by the nematicide Rugby 10% G even they reduced the final nematode population at the higher rate (0.2 g/pot) higher than that achieved by the nematicide Rugby 10% G. The obtained results are in accordance with previous reports which used the fungal and metabolites to control nematodes for a variety of agricultural applications on many different plants and fruits (Westerdahl *et al.*, 2005, Ami and Sipes 2008, Hagag, 2009, Khalil *et al*., 2010, Shawky *et al*., 2010 and Lewis 2017). Different mechanisms of nematodes inhibition by *M. verrucaria* were previously reported, those include affecting hatching of egg, inhibiting development, or through killing the nematode itself (Chavan *et al*., 2017 and Lamovšek *et al*., 2013). Warrior *et al*., (1999) reported that exposure to DiTera seemed to affect the neurosensory responses of nematodes adversely, eventually affecting motility and host/ mate finding behavior. In addition, *M. verrucaria* is known for its production of many enzymes such as lipases, chitinases, laccases, and proteinases (Wagenaar and Clardy, 2001).

Characterization of metabolites excreted by resulted in 13 compounds, according to the retention time and peak area percentage. Most of these compounds were found as fatty acids, organic acids, esters (Table 5). In the present study, the metaboilites extracted by *M. verrucaria* strongly inhibiteted egg hatching and J_2 of *M. incognita*. *M. verrucaria* isolates are known to produce metabolite compounds with antibiotic activity, which are reported as antagonists (Meyer *et al.*, 2004, Ruma *et al.*, 2014 and Nguyen *et al*., 2018). Interesting compound detected was 5,8 -Epoxy-15-nor-labdane which is a diterpene of the labdane family, *Atta-Ur-Rahman (1988)* reported that a variety of biological activities have been determined for labdane diterpenes including antibacterial, antifungal, antiprotozoal, and anti-inflammatory activities. Also, Thiocyanic acid, 2,4- dinitrophenyl ester was detected which is known to for its cardiotonic activities, insecticidal and nematicidel properties (Anyasor, *et al*., 2014). 14-.BETA.-H-PREGNA a kind of steroids known for a nematicidal activity or as plant resistance elicitors (Chitwood, 2002 and D'Addabbo, 2019). Nguyen *et al*., (2018) isolated and identified two macrocyclic trichothecenes verrucarin A and roridin A as major active metabolites of *M. verrucaria* which did not appear in GC-MS analysis in the present study. Elkhateeb and Daba (2019) reported that Roridins are lethal to some plants even in very small concentrations, and they cause myrotheciotoxicosis, sudden death in cattle and sheep accompanied with pulmonary congestion and edema, this investigation indicates that, the tested Egyptian isolate of *M. verrucaria* contains safe and harmless nematicidal compounds for the environment.

CONCLUSION

In the present study, the heat-sterilizing filtrate culture of *M.verrucaria* was strongly in inhibited the egg hatching and larvae of *M. incognita in vitro*. The study also indicated the efficiency of *M. verrucaria* formulae, which consists of the solids and soluble matter of the fungus, against root-knot nematode (*M. incognita*) on sugar beet plants under greenhouse conditions. Results of the present study provide evidence that the prepared formulae of *M. verrucaria* can be used as a feasible and safety bio-control nematicide for the biological control of *M. incognita* on sugar beet.

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تقييم النواتج الأيضية للفطر *Myrothecium verrucaria* كمبيد حيوى نيماتودى لمكافحة نيماتودا تعقد الجذور *Meloidogyne incognita* فى المعمل والصوبة على نباتات بنجر السكر

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فى هذه الدراسة، تم تقييم تأثير الراشح المعقم بالحرارة للفطر *Myrothecium verrucaria* ضد نيماتودا تعقد الجذور *Meloidogyne incognita* في المعمل، وتم تحضير حبيبات قابلة للذوبان من نواتج الأيض للفطر و اختبارها علي نباتات بنجر السكر المصابة بنيماتودا تعقد الجذور مقارنة بالمبيد الحيوي DiTera والمبيد الكيمياني راجبي G 10% تحت ظروف الصوبة. أظهرت النتائج المعملية أن راشح الفطر المعقم عن طريق المرشح السرنجة أو عن طريق الحرارة (التعقيم بالبخار) أدي لانخفاض معنوي في نسب فقس البيض وزيادة في عدد اليرقات الميتة وأعطي الراشح المعقم بالبخار عند تركيز 50% أعلي نسبة انخفاض في فقس البيض (94.23%) بينما الراشح المعقم بالمرشح كان أكثر فعالية في زيادة النسبة المئوية لليرقات الميتة (96.7%) بعد ثلاثة أيام من المعاملة، كما أنه لم يكن هناك فرق معنوي بين الراشح المعقم سواء عن طريق المرشح السرنجة أو التعقيم بالحرارة في تأثيرهما على تقليل نسب فقس البيض أو زيادة نسبة اليرقات الميتة من النيماتودا *M. incognita*. أظهرت نتائج تجربة الصوبة الزراعية أن جميع المعاملات المختبرة باستخدام التركيبة الفطرية المحضرة لفطر *M. verrucaria* أوباستخدام DiTera عند المعدلات المختلفة قللت بشكل كبير من عدد العقد على جذور بنجر السكر وأعداد النيماتودا النهائية في التربة مقارنة بالكنترول (النيماتودا فقط). حقق المعدل الأعلى (0.2 جم / اصيص) أعلى انخفاض في تعقد الجذور (86٪) بمعاملة DiTera وأعلى انخفاض في عدد اليرقات فى التربة باستخدام التركيبة الفطرية المحضرة للفطر (92.2٪). لم يكن تأثير هذه المعاملات مختلفًا بشكل كبير عن تأثير المبيد راجبى G 10%، كما لوحظ تحسن في نمو نباتات بنجر السكر من خلال المعاملات وخاصة معاملات التركيبة المحضرة لفطر *M. verrucaria*. تبين من التحليل باستخدام مقياس الطيف الكتلي للغاز (GC-MS) للنواتج الأيضية للفطر على أنها تحتوي على مركبات معروفة مضادة للميكروبات والنيماتودا. تقدم هذه الدراسة دليلاً على أن التركيبة المحضرة لفطر *M. verrucaria* يمكن استخدامها كمبيد حيوى آمن ضد نيماتودا تعقد الجذور *M. incognita* على نباتات بنجر السكر.