THE ROLE OF ANTAGONISTIC METABOLITES IN CONTROLLING ROOT-KNOT NEMATODE, *MELOIDOGYNE ARENARIA* ON TOMATO

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ABSTRACT

The effects of culture filtrates of Aspergillus niger, A. terreus and Trichoderma viride, citric or oxalic acids and the nematicide Vydate® L 24% on egg-hatching, second stage juveniles activity and the pathogenicity of Meloidogyne arenaria on tomato plants cv. Super strain B were studied under laboratory and greenhouse conditions. Treatments with the culture filtrate of the tested fungi and different dilutions of citric and oxalic acids caused significant inhibition (71.7-93.6%) in egg-hatching and (78.2-88.8%) in juveniles activity of M. arenaria. Soil treatment with culture filtrates of A. niger and T. viride, citric and oxalic acids and the nematicide Vydate® L 24% caused significant decrease (63.2-94.9%) in the number of nematode root galls and egg-masses developed on tomato plants compared to check treatment. Dry weight of root systems was significantly decreased due to M. arenaria infection. However, treatments with culture filtrates of the tested fungi, the two acids and Vydate® L 24% gave significant increase in the dry weight of shoot and root systems compared to check treatment.

***Key Words**: - Aspergillus niger - Aspergillus terreus - Trichoderma viride - citric acid - oxalic acid - Vydate® L 24% - Oxamyl- Meloidogyne arenaria – Antagonistic metabolites- Culture filtrates-Tomato plants.*

INTRODUCTION

Certain species of root-knot nematodes (*Meloidogyne arenaria, M. incognita, M. javanica*) are very important plant parasites affecting tomato production in most vegetable-growing regions in Egypt (Ibrahim and Rezk, 1988). In the past, control of these nematodes was done chemically using soil fumigants or systemic nematicides (Mostafa, 2000; Gugino *et al.*, 2006). Searching for microbial strains as sources of biological nematicides is an important goal to reduce the significant economic damage caused by plant-parasitic nematodes and to avoid the hazards of the chemical nematicides. Certain fungi exhibit a range of specificities and modes of action in their antagonistic activities toward nematodes, offering potential candidates to test against plant-parasitic nematodes (Nitao, *et al.*, 1999; Hussain *et al.*, 2001; Meyer *et al.*, 2004). Like other microbes, fungi can directly parasitize nematodes or secrete nematicidal metabolites or enzymes that affect nematode viability, which is an eco-friendly approach than the toxic chemicals. Toxic and inhibitory effects of several fungal filtrates against nematodes have been studied and confirmed (Mostafa, 1992; Zareen *et al.*, 1999; Ayoub *et al.*, 2000; Zareen *et al.*, 2001; Masadeh *et al.*, 2004; Siddiqui *et al.*, 2004 and Eapen *et al.*, 2005).

The biological response of plant parasitic nematodes to secondary metabolites produced by soil fungi is of interest because of the possible effects occurring during plant parasitism and their potential exploitation as pesticides of natural origin (Zaki, 1999; Ayoub et al., 2000 and Hussain et al., 2001).

Many microorganisms such as fungi can produce a variety of fungal metabolites, termed mycotoxins, which affect adult nematode activity and inhibit egg-hatching and juvenile development. These mycotoxins may include many organic acids such as citric and oxalic acids that produced by *Aspergillus niger* and *Trichoderma viride* in their culture media (Zuckerman et al., 1994; Siddiqi et al., 2001 a&b; Haq et al., 2002).

The objectives of the present study were to study the effects of culture filtrates of *Aspergillus niger, A. terreus* and *Trichoderma viride*, citric and oxalic acids on egg-hatching and second stage juvenile activity of *Meloidogyne arenaria* under laboratory as well as its infection on tomato plants under greenhouse conditions.

MATERIALS AND METHODS

Fungal culture:

Aspergillus niger Van Tegham, *Aspergillus terreus* Thom and *Trichoderma viride* Pers. ex Gray, were obtained from the culture collection of the Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Alexandria, Egypt.

Nematode inoculum preparation:-

Females and egg-masses of *M. arenaria* (Neal) Chitwood were isolated from infected tomato roots. Cultures of this nematode species were established from single egg-masses of adult females previously identified by the morphological characteristics of the female perineal patterns (Taylor and Sasser, 1978; Eisenback and Triantaphyllou, 1991) and reared on tomato plants (*Lycopersicon esculentum* Mill) cv. Super strain B in a greenhouse. The root-knot nematode eggs were extracted from infected tomato roots using sodium hypochlorite (NaOCl) solution as described by Hussey and Barker (1973). *M. arenaria* eggs were placed in sterile distilled water and the hatched 2nd stage juveniles (J_2) were used in the laboratory tests.

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Preparation of culture filtrates:-

The tested fungi *A. niger*, *A. terreus* and *T. viride* were grown in 250 ml Erlenmeyer flasks containing 100 ml of sterilized Czapek's Dox Liquid medium (**CDL**) supplemented with penicillin (100 units/l) and streptomycin (0.2 g/l) and inoculated with 3 discs of 0.5 cm diam. of actively culture of the test fungi grown on PDA medium. The pH of the medium was adjusted to 6.8 before sterilization. Flasks receiving only PDA discs served as control. The flasks were incubated at 27± 2 °C for 15 days. Each liquid culture was filtered through two layers of Whattman No.1 filter paper in a Buckhner funnel to remove any fungal spores (Siddiqui *et al.*, 2000). Culture filtrates obtained were designated as pure standard culture filtrates (**CF**), which were used further.

Effect of culture filtrates of *A. niger*, *A. terreus* and *T. viride* on egg-hatching and juvenile activity of *M. arenaria* under laboratory conditions:

The collected culture filtrates of the tested fungi were evaluated as **CF** or fifty percent dilutions (**CF/2**) which prepared by adding equal volume of sterile distilled water to the **CF** of the tested fungi to study their effect on egg-hatching and J_2 activity in two separate tests. Each test was done in 24-well tissue culture plates; each well received 2 ml of each test filtrate. A total of 85 nematode eggs/well were added in 20 µl of water, and 50 fresh J_2 of *M. arenaria* were added/well. Each treatment was replicated eight times. *M. arenaria* eggs or J_2 were placed in sterile distilled water and sterilized **CDL** medium for check treatment.

Effect of citric and oxalic acids on egg-hatching and juvenile activity of *M. arenaria* under laboratory conditions

Two separate tests were done to study the effect of citric and oxalic acids on egg-hatching and J_2 activity of *M. arenaria*. Three dilutions (2.5%, 5% and 10%) of citric and oxalic acids were prepared by dissolving citric and oxalic acids in sterile distilled water. Two ml of each acid dilution was placed in 24-well tissue culture plates. A total of 85 eggs/well were added in 20 µl of water, and 50 fresh J_2 of *M. arenaria* were added/well. Treatments were replicated eight times. Each of *M. arenaria* eggs or J_2 were placed in sterile distilled water and sterilized **CDL** medium for check treatment.

Observation on the effects of the fungi culture filtrates or citric and oxalic acids on egg-hatching and J_2 activity was taken at 48 hours after adding the nematode eggs and J_2 on culture filtrates or acid dilutions. The nematode J_2 were considered to be inactive when they did not move on probing with a fine needle.

Effect of culture filtrates of the tested fungi, citric, oxalic acids and Vydate® L 24% on *M. arenaria* infected tomato plants under greenhouse conditions:

Four-wk-old tomato seedlings cv. Super Strain B were transplanted into 20 cm diam. clay pots filled with autoclaved sandy clay soil (2:1 v:v), as one seedling/pot. Ten days later, fifteen pots were treated with **CF** of each *A. niger*, *A. terreus* and *T. viride* as 50 ml **CF**/pot. Another fifteen pots were treated with 50 ml of citric and/or oxalic acids (applied alone or mixed together) at the rate of 10% solution/pot. Five pots treated with 10% / pot of the nematicide, Vydate® L 24% (Oxamyl) produced by DuPont® Crop Protection, Wilmington, DE., USA. All treatments inoculated with 2000 *M. arenaria* eggs/pot.

Five pots, received sterile distilled water, and five pots received sterilized **CDL** medium were served as check. Each treatment was replicated five times. Pots were arranged in randomized complete block design and irrigated daily. The experiments were terminated 45 days after nematode inoculation. Dry weight of shoot and root systems, number of nematode root galls and egg-masses were determined.

Statistical analysis:

Data obtained were statistically analyzed according to SAS software program (SAS Institute, 1997). Data of the numbers of nematode root galls, egg-masses and juveniles were transformed to $\sqrt{x+1}$ before statistical analysis. Comparison among means was made via the least significant difference (LSD) at the 5% level of probability.

RESULTS

Effect of culture filtrates of *A. niger*, *A. terreus* and *T. viride* on egg-hatching and juvenile activity of *M. arenaria* under laboratory conditions

The effects of culture filtrates (**CF** and **CF/2**) of *A. niger*, *A. terreus* and *T. viride* on *M. arenaria* egg-hatching and J_2 activity were presented in Table 1. Treatments with **CF** of *A. niger* and *T. viride* caused (71.7-78.2%) inhibition in egg-hatching and J_2 activity while treatments with **CF/2** of these fungi showed 56.4-69.9% inhibition. On the other hand, treatments with **CF** and **CF/2** of *A. terreus* were less effective, caused respectively 64.4% and 48.4% inhibition in egg-hatching and J_2 activity of *M. arenaria* compared with check treatment (Table 1).

Effect of citric and oxalic acids on egg-hatching and juvenile activity of *M. arenaria* under laboratory conditions

Data in Table (2) showed the effect of citric and oxalic acids dilutions on *M. arenaria* egg-hatching and J_2 activity. Treatments with 2.5%, 5% and 10% of combined treatment with citric and oxalic acids caused the highest inhibition (79.6-93.6%) of J_2 activity and egg-hatching while treatments with 5% and 10% of each acid alone showed 57.4-78.8% inhibition. Treatment with 2.5% of each acid alone was less effective, showed 41.8-56.0% inhibition in J_2 activity and egg-hatching of *M. arenaria* compared with check treatment (Table 2).

Effect of culture filtrates of the tested fungi, citric, oxalic acids and Vydate® L 24% **on *M. arenaria* infected tomato plants under greenhouse conditions**

The effects of **CF** of *A. niger, A. terreus* and *T. viride*, 10% of citric and/or oxalic acid on *M. arenaria* infecting tomato plants were presented in Table 3. Treatments with Vydate® L 24%, citric and oxalic acids and **CF** of *T. viride* resulted in great reduction (88.8-94.9%) in the number of nematode root galls and egg-masses while treatments with **CF** of *A. niger* and citric acid showed 72.4-84.0% reduction. In addition, treatments with **CF** of *A. terreus* and oxalic acid caused 63.2-66.3% reduction in number of root galls and nematode egg-masses as compared to check treatment.

Dry weight of shoot and root systems decreased due to *M. arenaria* infection. However, treatments with different **CF** of all tested fungi, citric, oxalic acids and the nematicide gave significant increase in the dry weight of shoot and root systems. There was no significant difference between check treatments in number of nematode root galls and egg-masses or dry weight of shoot and root systems (Table 3).

Table 1. Effects of culture filtrates (CF) of *Aspergillus niger, A. terreus* and *Trichoderma viride* on egg-hatching and 2nd stage juvenile (J_2) activity of *M. arenaria* under laboratory conditions

Treatment	No. of hatched J_2 after 48 hours	Relative[x] hatching %	Hatching[y] Inhibition %	No. of active J_2 after 48hours	Activity[z] inhibition of J_2%
Nematode +Dist. Water	85.0 a	—	—	50.0 a	—
Nematode +CDL medium	84.4 a	*99.3*	*0.7*	49.8 a	*0.4*
Nematode +*A. niger* CF/2	25.6 d	*30.1*	*69.9*	18.9 d	*62.2*
Nematode +*A. niger* CF	18.5 f	*21.8*	*78.2*	10.9 e	*78.2*
Nematode+*A. terreus* CF/2	34.5 b	*40.6*	*59.4*	25.8 b	*48.4*
Nematode +*A. terreus* CF	30.3 c	*35.6*	*64.4*	22.6 c	*54.8*
Nematode +*T. viride* CF/2	27.4 d	*32.2*	*67.8*	21.8 c	*56.4*
Nematode +*T. viride* CF	24.1 e	*28.3*	*71.7*	13.6 e	*72.8*

Dist. Water +Nematode = Check. **CDL medium +Nematode** = Check.

CDL = Czapek's Dox liquid medium.

Nematode = 85 *M. arenaria* eggs /treatment for hatched experiment & = 50 active J_2/treatment for activity experiment.

[x] **Relative hatching %** = No. of hatched juveniles in each treatment / No. of hatched juveniles in check × 100

[y] **Hatching inhibition %** = 100 – Relative hatch (%).

[z] **Activity inhibition%**= $\frac{\text{No. of active juveniles in check - No. of active juveniles in each treatment}}{\text{No. of active juveniles in check}} \times 100$

Number of hatched and active juveniles was transformed to angular transformation before statistical analysis.

Data are averages of 8 replicates.

Values of the same column followed by the same letter are not significantly different at $P = 0.05$ of LSD test.

Table 2. Effect of citric and oxalic acids on egg-hatching and 2nd stage juvenile (J_2) of *M. arenaria* under laboratory conditions

Treatment	Dilution %	No. of hatched J_2 after 48hours	Relative[x] hatching %	Hatching[y] Inhibition %	No. of active J_2 after 48 hours	Activity[z] inhibition of J_2 %
Nematode + Dist. water	—	85.0 a	—	—	50.0 a	
Nematode+ Citric acid	2.5	37.4 c	*44.0*	*56.0*	27.6 c	*44.8*
	5	22.1 e	*26.0*	*74.0*	18.4 e	*63.2*
	10	18.0 f	*21.2*	*78.8*	14.8 f	*70.4*
Nematode + Oxalic acid	2.5	40.5 b	*47.6*	*52.4*	29.1 b	*41.8*
	5	24.1 d	*28.4*	*71.6*	21.3 d	*57.4*
	10	18.8 f	*22.1*	*77.9*	17.0 e	*66.0*
Nematode + Citric and Oxalic acids						
	2.5	10.1 g	*11.9*	*88.1*	10.2 g	*79.6*
	5	8.5 g	*10.0*	*90.0*	8.5 h	*83.0*
	10	5.4 g	*6.4*	*93.6*	5.6 i	*88.8*

Nematode + Dist. water= Check.
Nematode = 85 *M. arenaria* eggs /treatment for hatched experiment & = 50 active J_2/treatment for activity experiment.
[x] **Relative hatching %** = No. of hatched juveniles in each treatment / No. of hatched juveniles in check × 100
[y] **Hatching inhibition %** = 100 – Relative hatch (%).
[z] **Activity inhibition%**= No. of active juveniles in check -No. of active juveniles in each treatment × 100
No. of active juveniles in check
Number of hatched and active juveniles was transformed to angular transformation before statistical analysis.
Data are averages of 8 replicates.
Values of the same column followed by the same letter are not significantly different at $P = 0.05$ of LSD test.

Table 3. Effect of culture filtrates of the tested fungi, citric, oxalic acids and Vydate® L 24% on *M. arenaria* infected tomato plants under greenhouse conditions

Treatment	Number of galls/ root	Redaction %	Number of egg-masses/ root	Redaction %	Shoot dry weight (g)	Root dry weight (g)
Nematode alone (Check)	1024.4 a	—	1019.0 a	—	8.4 f	6.3 e
Nematode +CDL medium (Check)	1022.0 a	*0.2*	1017.4 a	*0.2*	8.2 f	6.5 e
Nematode +*A. niger* CF	173.8 e	*83.0*	163.0 e	*84.0*	24.0 c	19.5 b
Nematode +*A. terreus* CF	377.2 b	*63.2*	365.0 b	*64.2*	19.2 d	12.9 c
Nematode +*T. viride* CF	115.2 f	*88.8*	107.4 f	*89.5*	28.0 b	24.6 a
Nematode +Citric acid (10%)	283.0 d	*72.4*	270.4 d	*73.5*	19.5 d	10.2 d
Nematode +Oxalic acid (10%)	354.4 c	*65.4*	343.0 c	*66.3*	15.4 e	10.9 d
Nematode+Citric acid and Oxalic acid (10%)	99.2 g	*90.2*	94.4 g	*90.7*	30.5 a	23.9 a
Nematode + Vydate® L 24% (10%)	62.4 h	*93.9*	51.4 h	*94.9*	27.2 b	20.1 b

CDL = Czapek's Dox Liquid medium. **N = 2000 *M. arenaria* eggs and larvae /pot.**
Data are averages of 5 replicates.
Values of the same column followed by the same letter are not significantly different at $P = 0.05$ of LSD test.

DISCUSSION

Microorganisms which grown in soil rhizosphere are ideal for use as biocontrol agents since rhizosphere of many plants provides front line defense for roots against attack by soil-borne pathogens (Weller, 1988; Zareen *et al.*, 2001 and Siddiqui *et al.*, 2004). The present efforts of laboratory experiment showed that treatments with culture filtrate of *A. niger*, *A. terreus* and *T. viride*, different dilutions of citric and oxalic acids caused great reduction in egg-hatching and inhibited J_2 activity of *M. arenaria*. These findings are in agreement with those of other workers (Zuckerman *et al.*, 1994; Oduor *et al.*, 1996; Hussain *et al.*, 2001; Siddiqui *et al.*, 2001 b; Randhawa, *et al.*, 2001; Meyer *et al.*, 2004 and Sharon *et al.*, 2007).

Results of the present investigation revealed that treatments with culture filtrate of *A. niger* and *T. viride* and a mixture of citric acid and oxalic acid were more effective in reducing egg-hatching and inhibited J_2 activity of *M. arenaria* compared with other treatments. Previous and recent studies indicated the presence of citric acid and oxalic acid in the culture filtrates of certain fungi, *A. niger* and *T. viride* which effect on the root-knot nematodes (Vergano *et al.*, 1996; Ali *et al.*, 2001; Haq *et al.*, 2002 and Masadeh *et al.*, 2004).

Zuckerman *et al.* (1994) reported that *A. niger* culture filtrate suppressed the activity of *M. incognita* J_2 due to the presence of citric and oxalic acids as mycotoxins metabolites produced in culture filtrates of *A. niger*. Further, these two acids had synergistic action upon each other that help to understand the mode of action of *A. niger*.

In addition, in pot experiment, treatments with culture filtrates of the tested fungi or citric and oxalic acids and the nematicide Vydate® L 24% suppressed nematode infection and reproduction on tomato plants and enhanced the growth of tomato plants. These findings are in agreement with those of other workers (Vaishnav *et al.*, 1985; Mostafa, 2000; Siddiqui *et al.*, 2000; Hussain *et al.*, 2001; Eapen *et al.*, 2005 and Sharon *et al.*, 2007).

The presence of toxic metabolites in fungal culture filtrates has reported to reduce root-knot nematode infection and enhanced the growth of host plant (Zareen *et al.*, 1999; Zareen *et al.*, 2001 and Siddiqui *et al.*, 2001 b). Siddiqui *et al.* (2000) demonstrated that *A. terreus* posses thermostable nematicidal compounds and has the potential to control nematode population densities in soil and subsequent root-knot disease severity on tomato plants.

Based on the present data, culture filtrates of certain of *Aspergillus* spp. and *Trichoderma* spp. or extracted their toxic metabolites can be used as a safe method to reduce the destruction of root-knot nematodes infection on host plants.

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الملخص العربى

دور نواتج الأيض التضادية فى مقاومة نيماتودا تعقد الجذور *M. arenaria* على الطماطم

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تم دراسة تأثير راشح الفطريات *Aspergillus niger* و *A. terreus* و *Trichoderma viride* وحمض الستريك وحمض الاوكساليك و مبيد الفايدت ل ٢٤% على فقس بيض و حيوية الطور اليرقى الثانى والأثر المرضى لنيماتودا تعقد الجذور *Meloidogyne arenaria* على نباتات الطماطم صنف سوبر أسترين ب تحت ظروف المعمل و الصوبة. نتج عن المعاملة براشح الفطريات المختبرة والتخفيفات المختلفة لحامض الستريك و الاوكساليك حدوث تثبيط معنوي بمعدل ٧١,١ الى ٩٤,٦% فى فقس البيض و ٧٨,٢ الى ٨٨,٨% فى حيوية يرقات نيماتودا تعقد الجذور، كما أدت معاملة التربة براشح كلا فطر الـ *A. niger* و *T. viride* وحمض الستريك و الاوكساليك ومبيد الفايدت ل ٢٤% إلى حدوث نقص معنوى بمعدل ٦٣,٢ الى ٩٤,٩% فى أعداد العقد النيماتودية و أكياس البيض المتكونة على جذور نباتات الطماطم مقارنة بالمعاملة بالنيماتودا بمفردها. كما أدت اصابة نباتات الطماطم بنيماتودا تعقد الجذور إلى حدوث نقص معنوى واضح فى الوزن الجاف لكلا من المجموع الجذرى و الخضرى، فى حين نتج عن المعاملة براشح الفطريات و الأحماض العضوية المختبرة و المبيد النيماتودى حدوث زيادة معنوية فى الوزن الجاف لكلا من المجموع الجذرى و الخضرى مقارنة بالمعاملة بالنيماتودا بمفردها (الكنترول).