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EFFECT OF CULTURE FILTERATES OF THREE *Trichoderma* SPECIES, *Fusarium solani* AND *Rhizoctonia solani* ON EGG HATCHING AND JUVENILE MORTALITY OF *Meloidogyne incognita IN VITRO*

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ABSTRACT

Culture filtrates of three *Trichoderma* spp. (*T. album, T. harzianum* and *T. viride*), *Fusarium solani* (Mart.) Sacc., and *Rhizoctonia solani* Kühn have been found to cause adverse effects on egg hatching and juvenile mortality of the root-knot nematode, *Meloidogyne incognita* (Kofoid and White) Chitwood *in vitro*. The nematicidal activity was directly proportional to concentration of culture filtrates. *T. viride* gave the highest effect followed descendingly by *T. harzianum* and *T. album*. Moreover, the inhibitory effect of tested *Trichoderma* spp., culture filtrates on egg hatching was more pronounced when they were grown on gliotoxin fermentation medium (GFM) as compared to the parallel values when they were grown on potato dextrose broth (PDB) medium. Culture filtrates of *F.solani* and *R.solani* significantly reduced number of hatched juveniles and increased juvenile mortality of *M.incognita*. The inhibition of nematode hatching was significantly affected by fungal species, presence of fungal spores and mycelia in the filtrate and concentration used. Whereas the fungal species and concentration resulted significant effect on numbers of juveniles that exhibited no movement and counted as dead, while no observable differences were detected in filtrates contained spores and mycelia as compared to those free from spores and mycelia. Generally, *F.solani* resulted significant higher effects in abrupt egg hatching and increased juvenile mortality as compared to the respective values for *R.solani*.

Key words: *Trichoderma* spp., *Fusarium solani*, *Rhizoctonia solani*, *Meloidogyne incognita*, egg-hatching, juvenile mortality.

INTRODUCTION

Root-knot nematodes *Meloidogyne* spp. constitute a major group of plant pathogenic nematodes affecting crop production. Their world wide distribution, extensive host range and involvement with fungi and bacteria rank them among the top five major plant pathogens affecting the world's food supply (Sasser, 1979).

In Egypt, nematological studies revealed that root-knot nematodes cause severe damage to the majority of economic crops especially in localities with sandy soils (Ibrahim, 1985). For several decades, nematicides have been used to control these pests with remarkable results. However, concerns about public health and environmental safety have led to restrictions on chemical nematicide applications for nematode control. Therefore, development of alternative new environmentally methods such as biological control to enhance current management systems are very necessary (Meyer, 2003).

Many organisms including fungi, bacteria, soil invertebrates and predatory nematodes have been reported as biocontrol agents against plant parasitic nematodes (Stirling, 1991). Since fungi and nematodes occur concomitantly in the rhizosphere, the toxic metabolites naturally produced by these fungi might be responsible for keeping low levels of nematode populations.

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A lot of literature suggested that some species of *Trichoderma* have been used widely against root-knot nematodes (Singh *et al.*, 1983; Meyer *et al.*, 2000; Sharon *et al.*, 2001 and Sahebani & Hadavi, 2008). On the other hand, several attempts have also been done to assess the nematicidal effect of certain *Fusarium* spp. and/or *Rhizoctonia* spp. against root-knot nematodes (Mani & Sethi, 1984; Ali, 1989 and Zareen *et al.*, 2001).

Therefore, the objective of the present study was to evaluate nematicidal activity of three species of *Trichoderma i.e. T. album, T. harzianum* and *T. viride*, *Fusarium solani* and *Rhizoctonia solani* culture filtrates on egg hatching and juvenile mortality of *Meloidogyne incognita in vitro.*

MATERIALS AND METHODS

Preparation of Egg-masses and Second Stage Juveniles

The population of *Meloidogyne* species used in these experiments was obtained from infected eggplant roots collected from newly reclaimed sandy soil of El-Khatara Project, El-Sharkia Governorate .The species of nematode were identified after examination of 20 specimens based on morphology of perineal pattern of mature females and morphometry of second stage juveniles according to Taylor & Sasser (1978) and Eisenback *et al.* (1981). Perineal patterns were prepared based on the technique descrided by Taylor and Netscher (1974).

The galled roots of eggplant were soaked in tap water for one hour to remove adhering soil particles. Egg-masses needed for experiment were hand picked up with fine forceps from small galls. The obtained egg-masses were surface sterilized in 0.5% sodium hypochlorite (Chlorex) for 3 min., and quickly washed several times with sterile water to remove residues of NaOCl by passing the solution through a 200-mesh (75µm) sieve. The collected egg- masses were then refrigerated overnight at 5 °C and used next day for the assay (Hussey and Barker, 1973).

To obtain second stage juveniles, egg-masses were incubated in distilled water for 5 days at 28±2°C. Freshly hatched juveniles were separated daily using a micropipette after sedimentation of egg-masses.

Source of the Tested Fungi

Three isolates of *Trichoderma* species (*T. album, T. harzianum* and *T. viride*) were obtained from the Plant Pathology Lab. Agricultural Botany and Plant Pathology Department, Faculty of Agriculture, Zagazig University. On the other hand, pathogenic *Fusarium solani* (Mart.) Sacc. and *Rhizoctonia solani* Kühn were isolated from infected eggplant roots collected from El-Khatara Project, EL-Sharkia Governorate and identified according to Barnett and Hunter (1998) then confirmed by Assiut University Mycological Center (AUMC), Assiut University, Egypt.

Culture Filtrates of Tested Fungi and Bioagents

Isolates of *Trichoderma* species were grown on two liquid media *i.e.* potato dextrose broth (PDB) medium and gliotoxin fermentation medium (GFM). The last one is composed of ammonium tartarate, 2 g; $MgSO_4$,1 g; KH_2PO_4, 2 g; $FeSO_4$,0.01 g; dextrose, 25 g and distilled water 1000 ml (Brain and Hemming, 1945). GFM chemicals enhance the toxin secretion under dark conditions by the different *Trichoderma* spp. (Abd El-Moity and Shatla, 1981). On the other hand, pathogenic fungi *Fusarium solani* and *Rhizoctonia solani* were incubated for eight days at 28±4°C under darkness.

The liquid cultures of *Trichoderma* spp. were filtrated using sterilized filter paper Whatman No.1 twice under sterilized conditions to obtain spore and / or mycelia free culture filtrate and considered as culture filtrate. On the other hand, *F. solani* and *R. solani* culture medium of each fungus was divided into two parts, the first one was filtrated as implemented method with *Trichoderma* spp., while, the second part was filtrated using muslin cloth to remove big fragments of mycelia and obtain filtrates not completely free of mycelia or spores and considered as non culture filtrate (Naserinasab *et al.*, 2011). The obtained filtrates of the tested fungi were considered as 100% concentration. Further different dilution of 75, 50 and 25 % were prepared by adding request amount of sterilized distilled water.

Influence of Culture Filtrates on *Meloidogyne incognita* Egg Hatching

Ten egg-masses of uniform size were added to ten ml of each concentration (0, 25, 50, 75 and 100%) in 9 cm diameter Petri-dishes. The control treatment (0.0 concentration) was prepared using sterilized distilled water without culture filterate. Each treatment was replicated three times. All treatments were left in dark under room temperature at 28±4°C for two days after exposure. Numbers of hatched juveniles were counted using a research microscope (100X magnification). Hatching inhibition percentage was calculated in comparison with control treatment according to the following equation:

$$\text{Inhibation } \% = \frac{\text{Control - treatment}}{\text{Control}} \times 100$$

Influence of Culture Filtrates on *Meloidogyne incognita* Juvenile Mortality

Dilutions from each cultural filtrate (0, 25, 50, 75 and 100%) were prepared as mentioned before. Ten milliliters of each concentration were placed in 9 cm diameter Petri-dishes. About 100 newly hatched second stage juveniles in 0.2 ml of water were added to each Petri-dish. All Petri-dishes were kept at room temperature of 29±4 °C. The control treatment was 10 ml sterilized distilled water with the same number of juveniles. Three replicates were used for each treatment. Two days after treatment, others exhibited number of dead juveniles as those showing no movement (not active) and the active juveniles with normal movement were counted under 100X magnification. Mortality percent was calculated according to the following equation:

$$\text{Mortality } \% = \frac{\text{Dead juveniles}}{(\text{Live} + \text{dead}) \text{ juveniles}} \times 100$$

Statistical Analysis

Data were statistically analysed according to the procedures reported by Snedecor and Cochran (1980), using the computer program Costat version 6.400. F test as well as standard error (±S.E.) were calculated. Means were compared by Duncan's multiple range test at 5% (Duncan, 1955).

RESULTS AND DISCUSSION

Influence of Culture Filtrates of Three *Trichoderma* spp. on *Meloidogyne Incognita* Egg Hatching *In Vitro*

Observations on number of juveniles identified as *Meloidogyne incognita* (Kofoid and White) Chitwood, hatched after two days of exposure to four culture filtrates concentrations of three *Trichoderma* spp. (*T.album*, *T. harizinum* and *T. viride*) grown on two different media (PDB and GFM) are presented in Table 1. The analysis of variance revealed that *Trichoderma* spp., media and concentrations showed significant effect at $P < 0.05$ on egg hatching. Moreover, each dual interaction *i.e Trichoderma* spp. and media, *Trichoderma* spp. and concentrations and finally media and concentrations, was significant. However, the treble interaction between the three studies factors, was insignificant.

Data clearly indicated that culture filtrates of the tested fungi, inhibited hatching of *M. incognita*. The numbers of hatched juveniles were directly correlated with culture filtrates concentration.

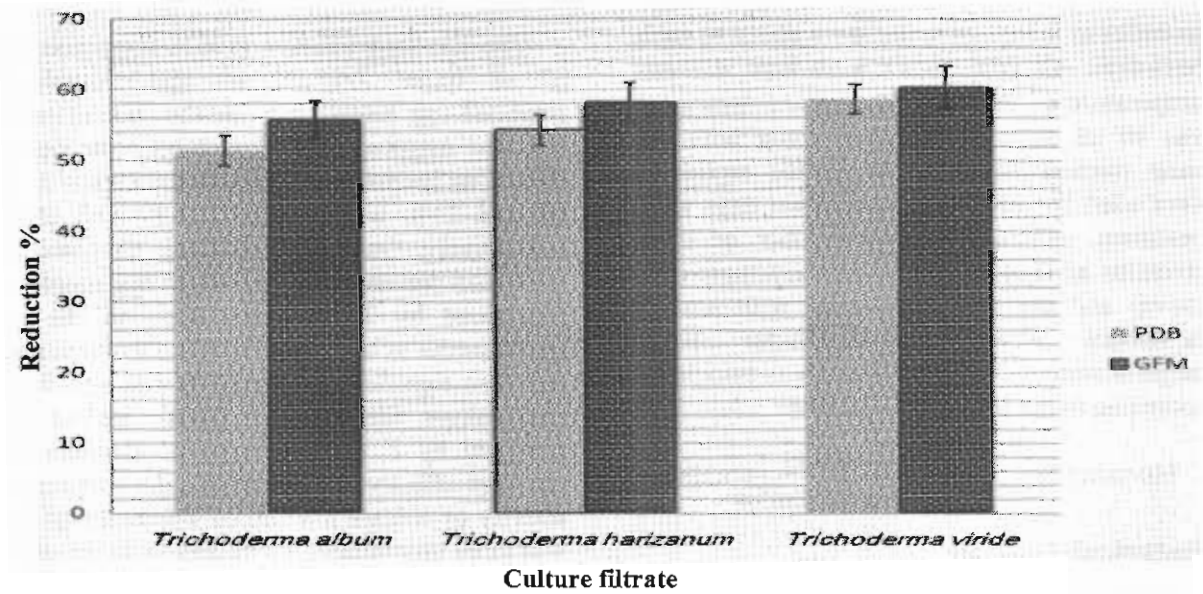
The standard solution (100% concentration) of all tested *Trichoderma* spp. completely inhibited egg hatching, while the least effective one was detected at the lower concentration of 25%. The hatched juvenile general means at 75, 50 and 25% were 33.06, 152.39 and 287.5, respectively. On the other hand, the hatching inhibition percent of *M. incognita* egg-masses as influenced by *Trichoderma* spp. on the two tested media as compared with control treatment was also significant (Fig. 1). It was also found that culture filtrate of *T. viride* ranked first followed by *T. harizanum* while *T.album* was the least effective. Furthermore, the suppressive activity of culture filtrates of *Trichoderma* spp. was more pronounced when they were grown on GFM medium compared with PDB medium. Means of reduction percent in egg hatching for culture filtrates of *T. album, T. harizanum* and *T. viride* grown on PDB and GFM media were 51.30,55.65%; 54.25, 58.12%; and 58.63, 60.27%,respectively. The obtained results came in agreement with those reported by many authors. They indicated that culture filtrates of

Table 1. Numbers of *Meloidogyne incognita* egg hatching as affected by four different concentrations of three *Trichoderma* species culture filtrate grown on two media *in vitro*

Concentration	*Trichoderma album*		*Trichoderma harzianum*		*Trichoderma viride*		General mean
	*PDB	**GFM	PDB	GFM	PDB	GFM	
Control	400.00	400.00	400.00	400.00	400.00	400.00	400.00[a]
25	316.66	304.00	297.33	282.33	262.33	262.33	287.50[b]
50	203.00	147.33	177.00	135.00	137.67	114.33	152.39[c]
75	54.33	35.67	40.67	22.33	27.33	18.00	33.06[d]
100	0.00	0.00	0.00	0.00	0.00	0.00	0.00[e]
Mean	194.80[A]	177.40[B]	183.00[A]	167.93[B]	165.47[A]	158.93[B]	
Reduction %	51.30[A]	55.65[B]	54.25[A]	58.12[B]	58.63[A]	60.27[B]	
General Mean	186.1[C]		175.5[B]		162.2[A]		174.59

*PDB denotes to potato dextrose broth medium. **GFM denotes to gliotoxin fermentation medium.

Notes: - Each value represents mean of three replicates for number of hatched juveniles.
-Means not followed by the same small letter in columns or capital letter in rows are significantly different at $P<0.05$.



PDB denotes to potato dextrose broth medium. GFM denotes to gliotoxin fermentation medium

Fig. 1. Reduction percent in hatching of *M. incognita* egg-masses as influenced by culture filtrates of three *Trichoderma* spp. grown on two media

Trichoderma spp., significantly inhibited egg hatching and increased juvenile mortality of *Meloidogyne* spp. (Singh *et al.*, 1983; Meyer *et al.*, 2000; Sharon *et al.*, 2001; Sahebani & Hadavi, 2008; Bokhary, 2009; Yang *et al.*, 2010 and Naserinasab *et al.*, 2011). In this respect, Sharon *et al.*, 2001 explained mechanisms which can be involved in suppressing root-knot nematodes by *Trichoderma* spp., as mycoparasitism, antibiosis, tolerance to stress through enhanced root and plant development, induced resistance, solubilization and sequencing of inorganic nutrients, inactivation of the nematode enzymes and enzymatic hydrolysis.

It is well known that, chitin is a common structural component (40% w/w) of a nematode egg shell (Wharton, 1980). Whereas, *Trichoderma* spp. are known to have proteolytic and chitinous activities which cause alteration in cuticular structure of eggs, changes in egg shell permeability or cause perforation in the cuticle which allows seepage of toxic metabolites into the eggs and cause physiological disorders. These factors might have an important role in the inhibition of egg hatch (Jatala *et al.*, 1985 and Lopez-Llorca, 1990). However, Meyer *et al.* (2000) showed that lack of detectable chitinase and protease activities from culture filtrates of *T. virens* and *Burkhalderia cepacia* suggested that the inhibitory factors in the *in vitro* assays were non-enzymic. Furthermore, antibodies that bind to *M. javanica* surface served as a tool for further investigation of the fungal attachment to nematodes, antibodies were found to improve parasitism *in vitro* (Sharon *et al.* 2009).

Effect of Culture Filtrates of Three *Trichoderma* Species on Juvenile Mortality of *Meloidogyne Incognita In Vitro*

Mortality of second stage juveniles of *M. incognita* as influenced by culture filtrates of *Trichoderma* spp., grown on two media using four concentrations are shown in Table 2. It was found that, culture filtrates of all the tested fungi were toxic to juveniles with varying degrees. Significant variations were observed between culture filtrates of the tested fungi. Similar results were also obtained, between the two media. Means of percent juvenile mortality with culture filtrates of *T. album, T. harizanum* and *T. viride* on PDB and GFM media were 64.21,69.89%; 63.58,69.68%; and 64.28,66.46%, respectively. Concentrations tested of the culture filtrates showed significant effect at $P< 0.05$ on juvenile mortality. A steady increase in juvenile inactivity was detected with increasing tested concentration of culture filtrates. Mobility of juveniles was completely inhibited at the highest concentration at 100 % compared with the three tested concentrations of the three different fungal filtrates grown on both media. Meanwhile, similar result was obtained at 75% concentration when the tested fungi were grown on GFM medium.

Table 2. Mortality percentage of *Meloidogyne incognita* juveniles as affected by different concentrations of three *Trichoderma* species culture filtrate grown on two media *in vitro*

Concentration	*Trichoderma album*		*Trichoderma harzianum*		*Trichoderma viride*		General Mean
	*PDB	**GFM	PDB	GFM	PDB	GFM	
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00^{a}
25	42.81	61.05	45.61	63.86	45.26	48.07	51.11^{b}
50	78.95	88.42	75.09	84.56	80.35	84.21	81.93^{c}
75	99.29	100.00	97.19	100.00	95.79	100.00	98.71^{d}
100	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{e}
Mean	64.21^{A}	69.89^{B}	63.58^{A}	69.68^{B}	64.28^{A}	66.46^{B}	
General Mean	67.05^{A}		66.63^{A}		65.37^{B}		66.35

*PDB denotes to potato dextrose broth medium. **GFM denotes to gliotoxin fermentation medium.

-Data followed by the same small letter in columns or capital letter in rows are not significantly differed at $P<0.05$.

General means of mortality percent as influenced by culture filtrates of the tested fungi at 25, 50, and 75% concentrations were 51.11, 81.93 and 98.71%, respectively.

The present findings revealed that *Trichoderma viride* gave the best results against *M. incognita* followed by *T. harzianum* while, T. *album* was the least effective. A similar effect was observed by Narasimhamurthy *et al.* (2011), who reported that *T. viride* was superior over other five bioagents included *T. harzianum.* Moreover,Singh *et al.* (1983) revealed that highest mortality of *M. incognita* occurred in culture filtrates of *T. viride* presumably because of the presence of higher amount of phenols. Contrarily, Bokhary(2009) showed that among five tested *Trichoderma* spp., on eggs and juveniles of *M. javanica* and *Rotylenchulus reniformis in vitro* , *T. harzianum* gave the favorite results followed descendingly by *T. hamatum, T. konginii, T. viride and T. reesei* . On the other hand, the nematicidal activity of *Trichoderma* spp., grown in GFM was more pronounced as compared to those grown on PDB medium. This might be due to GFM role in enhancing the toxin secretion by the tested fungi (Abd El-Moity & Shatla, 1981).Generally, present findings might throw some light on explaining the mechanisms involved into the diminishing of nematode population due to application of organic amendments which lead to marked increase in saprobic fungi in the rhizosphere.

Egg Hatching and Juvenile Mortality of *Meloidogyne incognita In Vitro* as Affected by Culture Filtrates of *Fusarium solani* and *Rhizoctonia solani*

Culture filtrates of *F.solani* and *R.solani* obviously reduced numbers of *M. incognita* hatched juveniles after two days of exposure as compared to water control as shown in Table 3. The inhibition of nematode hatching was significantly affected at $P<0.05$ by fungus species, presence of spores and mycelia in filtrates and the concentration used. The interactions between these factors were also significant. On the other hand, tested fungus species and concentrations gave significant effect on numbers of juveniles that exhibited no perceptible movements and counted as dead, while no observable differences were detected between filtrates having spores and mycelia as compared to those spores and mycelia free filtrates.

The tested species differed significantly in their ability to abrupt egg hatching and increase juvenile mortality. *F.solani* gave higher significant effects at $P<0.05$ on egg hatching and juvenile mortality as compared to the parallel values for *R.solani*. General means of egg hatching reduction percent of *F. solani* and *R. solani* were 66.77and 58.46 %, respectively, while the parallel values for juvenile mortality were 53.03 and 46.23 % respectively. On the other hand, culture filtrates of the two genera having, spores and mycelia yielded significantly less hatched juveniles as compared to filtrates without spores and mycelia with inhibition percent 67.7 and 65.9 % for *F. solani* and 59.6 and 57.3 % for *R. solani,* respectively, while the presence or absence of spores and mycelia showed non- significant effect on juvenile mortality with percent mortality being 53.8 and 52.3% for *F. solani* and 45.9 and 46.6 % for *R. solani*, respectively.

As the concentration of culture filtrates increased there were significant progressive increments in percentage of egg hatching inhibition and juvenile mortality. General means of reduction percent in egg hatching and juvenile mortality at 25, 50 and 75% concentrations for *F. solani* and *R. solani* were 61.3,20. 3%; 68.6, 46.4% and 83.2, 81.5%, respectively. Whereas, at 100% concentration the eggs within egg-masses failed in hatching and the mortality of juveniles were completely ceased after two days of exposure to culture filtrates of the tested fungi.

Culture filtrate of *Fusarium solani* has been found to cause adverse effects on hatching and juvenile mobility of *Meloidogyne* spp. (Mani &Sethi, 1984 and Zareen *et al.*2001). A number of active nematicidal compounds were reported from culture filterates of *Fusarium* spp. Moniliformin, fusarenone, neosolaniol, T2-toxin, verrucarin A and cytocholasin B produced by *Fusarium* spp. and other soil fungi reduced hatching and viability of *M. javanica* juveniles (Ciancio,1995). Non-polar, long-chain alkanes were tentatively identified as the nematicidal components of the *F. solani* culture filtrate (Mani *et al.*, 1986). Moreover, many *Fusarium* spp. were reported as toxin producers (Marasas *et al.*, 1984 and Ciancio, 1995).

Table 3. Egg hatching and juvenile mortality of *Meloidogyne incognita* as affected by four different concentrations of *Fusarium solani* and *Rhizoctonia solani* culture filtrate with or without spores and mycelia *in vitro*

Concentration	Hatching inhibition %				Mean	% juvenile mortality				Mean
	F.solani		*R.solani*			*F.solani*		*R.solani*		
	1	2	1	2		1	2	1	2	
Control	0	0	0	0	**0.0** [a]	0	0	0	0	0.0 [a]
25	71.78	70.42	56.25	46.79	**61.3** [b]	21	20.33	22.67	16.9	20. 3 [b]
50	77.4	74.9	61.66	60.32	**68.6** [c]	54.67	50.33	41.07	39.67	46.4 [c]
75	89.17	84.07	80.32	79.28	**83.2** [d]	93.33	90.67	65.67	76.33	81.5 [d]
100	100	100	100	100	**100** [e]	100	100	100	100	100 [e]
Mean	67.7 [A]	65.9 [B]	59.6 [A]	57.3 [B]		53.8 [A]	52.3 [A]	45.9 [A]	46.6 [A]	
General mean	66.77 [B]		58.46 [A]		62.62	53.03 [A]		46.23 [B]		49.6

1:Culture filtrates with spore and mycelia. 2: Culture filtrates without spore and mycelia.
- Values followed by the same small letter in columns or capitals letter in rows are not significantly differed at 5%.

Sakhuja *et al.* (1978) showed that culture filtrates of *F.solani* and *R.solani* appeared to have some toxic substances which inhbited hatching of *M. incognita in vitro*. Meanwhile, Ali (1989) indicated that culture filtrates of *R.solani* demonstrated toxic effect on eggs and juveniles of *M. incognita*.

The presents results showed that *F. solani* gave significantly higher effects in reducing egg hatching and juvenile movement as compared to *R. solani*. Anwar (2004) came to the same conclusion. He reported that the variation in hatching of *M. incognita* to culture filtrates of fungi may be due to the differences in the nature of their toxic metabolites.

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تأثير الرواشح المزرعية لثلاثة أنواع من الترايكودرما وفطري فيوزاريم سولاني ورايزوكتونيا سولاني علي فقس البيض وموت الأطوار اليرقية لنيماتودا ميلودوجينا انكوجنيتا تحت ظروف المعمل

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تكشف الدراسة الحالية تأثير الرواشح المزرعية لثلاثة أنواع من فطر الترايكودرما (ترايكودرما البم وترايكودرما هرزيانم وترايكودرما فيردي) وفطري فيوزاريم سولاني وريزوكتونيا سولاني علي فقس البيض وموت الاطوار اليرقية لنيماتودا تعقد الجذور (ميلودوجينا انكوجنيتا) في المعمل حيث كان التأثير الإبادي علي النيماتودا يتناسب طرديا مع تركيز رواشح البيئات. ومن جهة اخري وجد ان هناك تباين معنوي علي فقس البيض بين الأنواع الثلاثة من الترايكوديرما المختبرة حيث كانت ترايكودرما فيردي اعلاهم تأثيرا تليها ترايكودرما هارزيانم ثم ترايكودرما البم، علاوة علي التأثير المثبط لرواشح البيئات لفطريات الترايكودرما علي فقس البيض. فقد كان التأثير الاكثر وضوحا عند تنمية الفطريات علي بيئة تخمر الجليكوتوكسن (GFM) مقارنة بتنميتها علي بيئة مرق البطاطس المغذي (PDB). كان لرواشح مزارع الفيوزاريم سولاني والرايزوكتونيا سولاني تثبيط معنوي للأطوار اليرقية الفاقسة حيث ازداد موتها كما أثرت ايضا بدرجة كبيرة علي النيماتودا الفاقسة بإختلاف نوع الفطر وأيضا بإحتواء الرواشح علي ميسليوم الفطر وجراثيمه إذ أبدت الأنواع الفطرية وتركيزات رواشحها تأثيرا معنويا علي أعداد الأطوار اليرقية التي أظهرت عدم القدرة علي الحركة واحتسبت ميتة. هذا وقد أعطي فطر فيوزاريم سولاني أعلي مستوي تأثير معنوي علي فقس البيض وزيادة موت الأطوار اليرقية مقارنة بفطر رايزوكتونيا سولاني.

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