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EFFECT OF NEMATODE-TRAPPING FUNGI, *TRICHODERMA HARZIANUM* AND *PSEUDOMONAS FLUORESCENS* IN CONTROLLING *MELOIDOGYNE* SPP.

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Abstract

The culture filtrates of seven species of nematode-trapping fungi (NTF) (*Arthrobotrys conoides*, *A. cookedichison*, *A. eudermata*, *A. microscaphoides*, *A. oligospora*, *A.t haumasia* and *Clonostachys rosea*) in combination with *Trichoderma harzianum* and *Pseudomonas fluorescens* were evaluated for their potential against hatching eggs and second stage juveniles (J2) mortality of the root-knot nematode *Meloidogyne* spp. The combined treatment of *T. harzianum*, *P. fluorescens* and one of nematode-trapping fungi species showed better biocontrol activity comparing with that of using one of NTF species either alone or in combination with *T. harzianum* or *P. fluorescens*. Single treatment of *C. rosea* filtrate showed the lowest hatching eggs percentage (59.73%), while single treatment of *P. fluorescens* filtrates showed the highest percentage of J2 mortality (45.83%). The combined treatment of *P. fluorescens* and *T. harzianum* filtrates revealed low hatching eggs and high J2 mortality percentage (40.74% and 60.18% respectively). The combined treatment of *C. rosea*, *P. fluorescens* and *T. harzianum* filtrates gave the lowest hatching eggs rate (26.85%) and the highest J2 mortality of about 75%. It is found that treatment duration of 72 hours is the best to get effective reduced hatching eggs and J2 mortality.

Key words: *P. fluorescens*, *T. harzianum*, *Meloidogyne* spp., Nematode-trapping fungi, biocontrol agents (BCAs)

Introduction

The root-knot nematodes *Meloidogyne* spp are sedentary endoparasites, which are considered as the most dangerous in the world, attacking a wide range of plant families (Barker *et al.*, 1985; Gouveia *et al.*, 2017). In addition to their involvement with other pathogens, especially fungi in developing a lot of pathological complications which are hard to treat, they break and reduce plant resistance to other diseases and increases the likelihood of plant infection by other organisms (Turatto *et al.*, 2018). It is known that synthetic nematicides cause many problems such their toxicity to wide range of soil organisms, and appearance of resistant strains among nematodes, though they are still used broadly to control plant-parasitic nematodes (Dong and Zhang, 2006). Thus, there is an urgent need to find eco-friendly solutions which lead eventually for application of biocontrol agents. Fungi and bacteria are among various kinds of species being used as biocontrol agents (Waghunde *et al.,* 2016). Although, there are many biocontrol agents (BCAs) have been reported as potential and environmentally safe pesticides, only few of them have been commercialized (Meyer *et al.,* 2001; Roberts *et al.*, 2005).

Trichoderma species were evaluated for their efficiency in controlling the root-knot nematode *M.javanica*. Their nematicidal activity is attributed to variety of mechanisms such as mycoparasitism, antibiosis and competition (Howell, 2003; Vinale *et al.*, 2008). All mechanisms, except the competition one, are commons in nematode biocontrol process (Sharon *et al.,* 2001). *Trichoderma* species have many characteristics, such as production of natural compounds or secondary metabolites which could be volatile or non-volatile in nature. The function of these secondary metabolites is often obscure or unknown role, but with considerable importance to humankind in medical, industrial or agricultural applications (Bhardwaj and Kumar, 2017).

Jansson & Lopez-Llorca (2001) reported that

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nematophagous fungi are common soil inhabitants and infect living nematodes using different strategies. Nematode trapping fungi, one group of nematophagous fungi, form special structures that trap nematodes, some trapping fungi can produce nematicidal compounds when capture nematodes (Muhsin and Ali, 1998). Many species of nematode trapping fungi have an ability to produce nematicidal compounds such as linoleic acid, oligosporon and arthrobotrisin A (Stadler *et al.,* 1993; Anderson *et al.,* 1995; Anke *et al*., 1995; Wei *et al.,* 2011).

Some strains of *Pseudomonas* species are aggressive colonizers of the rhizosphere of different plants and have a wide range of antagonistic activity against plant pathogens, using different mechanisms such as antibiosis (Upadhyay *et al*., 1991), siderophore production (Winkelmann and Drechsel 1997) and nutrition or site competition (Bull *et al*., 1991).

Using single fungal or bacterial biocontrol agent (BCA) may cause many problems of biocontrol measures resulting insufficient control of the target nematodes. Applying combination of various BCAs may lead to increase biocontrol activity (Szabó *et al.,* 2012). Therefore, under a wide range of environmental conditions, combined BCA may give better variety of biocontrol effects (Meyer *et al.,* 2001; Roberts *et al.,* 2005). In addition, the combined activity of nematicides which produced by microorganisms could control wider spectrum of pests. Some of these combinations are not successful due to their incompatibility or poor antagonistic properties (Reaves and Crawford, 1994); however, numerous successful combined BCAs have been reported (Meyer and Roberts, 2002).

Szabó *et al.,* (2012) used different combinations of *Trichoderma* species and species of nematode-trapping fungi in order to control plant-parasitic nematodes. The present study investigates the effects of applying various combinations of three BCAs (nematode-trapping fungi, *T.harzianum* and *P.fluorescens*) against eggs hatching and second stage juveniles (J2) of *Meloidogyne* spp.

Materials and methods

Microorganisms

The filtrates of liquid cultures from *A. conoides* (Ac), *A. cookedichison* (Ack), *A. eudermata* (Ae), *A. microscaphoides* (Am), *A. oligospora* (Ao), *A. thaumasia* (At), *C. rosea* (Cr) (were isolated from agricultural soil in Miasan province/southern Iraq), *T. harzianum* (Th) and *P. fluorescens* (Pf) (were supplied by the Plant Protection Department/Agricultural College/ Basrah University) were tested either separately or in combination in order to evaluate the most appropriate partners against eggs hatching and second stage juveniles (J2) of the root-knot nematode *Meloidogyne* spp.

Preparation of culture filtrates of microorganisms

Nematode-trapping fungi (NTF) species were grown in corn mealbroth (CMB) (Sigma-Aldrich), *T. harzianum* was grown in potato dextrose broth and *P. fluorescens* was grown in the broth nutrient medium with the agitation rate of 120 rpm at 27°C for 10 days (72 hours for *P.fluorescens*), centrifuged at 3000 cycles for 15 minutes. Fungal and bacterial cultures were filtered on Whatman No.1 filter paper. The filtrates were sterilized using millipore (22μ).

Preparation of eggs and J2 of the second phase of *Meloidagyne* spp

Appropriate amounts of *Abelmoschus esculentus* roots infected with *Meloidagyne* spp were collected from different regions in Maysan province/southern Iraq. Eggs and second-stage juveniles (J2) were prepared according to Hussey and Barker (1973). Eggs were separated from egg masses with sodium hypochlorite (0.5%, 2 min) and hatched in water to produce infective J2.

Effect of single filtrates of NTF, *P. fluorescens* and *T. harzianum*

1ml (20-25 eggs or J2 of *Meloidogyne* spp) was added to a 5cm Petri dish followed by addition of 3ml of either one species of NTF or *P. fluorescens* or *T. harzianum* filtrates (triplicate sets), 3ml of sterile distilled water was added as control, the dishes were incubated at 28°C, the percentage of hatching eggs and J2 mortality were counted after 24, 48 and 72 hours.

Effect of combined filtrates of *P.fluorescens* or *T.harzianum* and NTF

1.5 ml of either *P. fluorescens* or *T. harzianum* in combined with 1.5ml of each species of NTF filtrates, the data were determined as above.

Effect of combined filtrates of *P. fluorescens*, *T. harzianum* and NTF

1ml of each of *P. fluorescens* and *T. harzianum* in combined with 1 ml of each species of NTF filtrates, the data were determined as above.

Data were statistically analyzed using analysis of variance (ANOVA), and treatments means were separated by Fisher's least significant difference (LSD)

Results

The combined filtrates of NTF, *P. fluorescens* and *T. harzianum* showed the highest biocontrol activity against hatching eggs and J2 mortality of the root-knot

nematode *Meloidagyne* spp then followed by the mixture of one species of NTF and either *P. fluorescens* or *T. harzianum*. On other hand, it seems that treatment for 72 hours lead to the lowest hatching eggs and highest J2 mortality.

It was found that treatment with single filtrates of NTF, *P. fluorescens* and *T. harzianum* suppressed the hatching eggs and increased J2 mortality of *Meloidagyne* spp comparing with control. The results showed an obvious correlation between efficiency and the type of microorganism. *C. rosea* filtrate caused the lowest hatching eggs percentage (59.73%), which was increased slightly to (60.16%) when applying *A. cookedichison* filtrate, while the highest value appeared when using *A. conoides* filtrate (65.28%) comparing with control (87.53%). The effect of the treatment duration was studied also, which showed decreasing in egg hatching percentage and increasing in the mortality of J2 over the time. Thus, the lowest eggs hatching percentage appeared at 72 h which is 50.89%, while the highest appeared at 24 h (79.55%) (Fig. 1). The maximum percentage of J2 mortality was 45.83% which obtained by using *P. fluorescens* filtrate followed by *C. rosea* (43.05%), while *A. conoides* filtrate caused the lowest percentage of J2 mortality comparing to control which was 16.66%. On the other hand, there is a positive correlation between J2 mortality and duration of treatment, thus, highest rate of J2 mortality was 52.9% after 72 h of treatment, followed by 37.49% after 48h (Table 1).

Fig. 2 explains that the combined filtrates of NTF species and *P. fluorescens* gave better results than that of single filtrate of either of them. Th+Pf filtrates gave the lowest hatching eggs percentage of 40.74%, followed by Cr+Pf filtrates 44.87%, while the highest hatching eggs percentage which caused by Ao+Pf filtrates was 50.94%. Hatching eggs percentage reached the highest 62.77% after 24h of treatment then decreased to 40.84% after 72h (Fig. 2).

On the other hand, Th+Pf filtrates were more efficient in enhancing J2 mortality (60.18%), followed by At+Pf filtrates 57.87%, while the lowest percentage of J2 mortality was observed when using Ack+Pf filtrates 52.78%. The percentage of J2 mortality was increased from 38.48% at 24 h to 75.83% at 72h of treatment (Table 2).

The combined filtrates of NTF species and *T.*

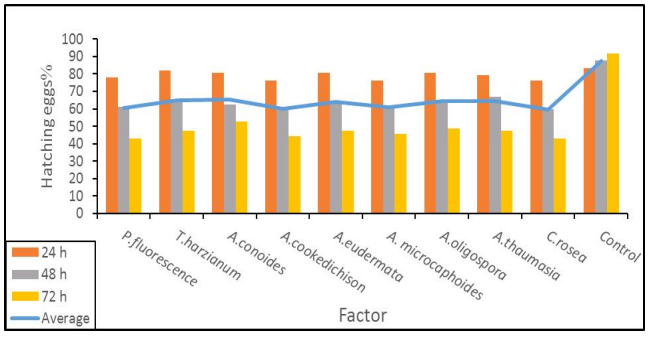

Fig. 1: Effect of single filtrates of NTF species, *P. fluorescens* and *T. harzianum* on hatching eggs percentage

Table 1: Effect of single filtrates of NTF species, *P. fluorescens* and *T. harzianum* on J2 mortality percentage

Influencing factor	Time (h)			Average
	24	48	72	
P. fluorescence	27.77	49.99	59.72	45.83
T. harzianum	18.06	34.7	58.33	37.03
A. conoides	19.44	37.49	47.22	34.72
A. cookedichison	23.61	40.27	59.72	41.2
A. eudermata	19.44	36.1	55.55	37.03
A. microcaphoides	26.39	43.04	58.33	42.59
A. oligospora	19.44	36.1	54.16	36.57
A. thaumasia	20.83	33.32	55.55	36.57
C. rosea	26.39	44.44	58.33	43.05
Control	12.5	16.66	20.83	16.66
Average	21.08	37.49	52.9	

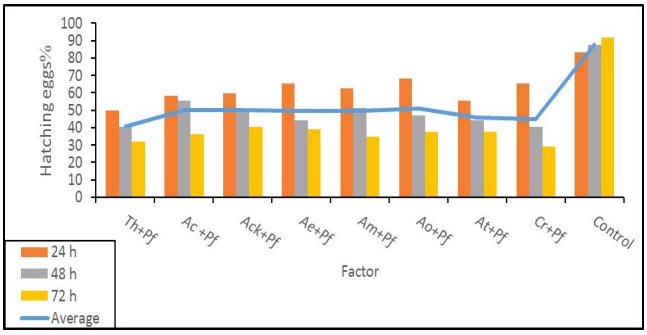

Fig. 2: Effect of NTF species in combination with *P. fluorescens* filtrates on hatching eggs percentage

Table 2: Effect of NTF species in combination with *P. fluorescens* filtrates on J2 mortality

Influencing factor	Time (h)			Average
	24	48	72	
Th+Pf	45.83	61.11	73.61	60.18
Ac+Pf	41.66	44.45	73.61	53.24
Ack+Pf	40.28	52.78	65.27	52.78
Ae+Pf	34.7	55.55	70.83	53.69
Am+Pf	37.5	52.78	70.83	53.7
Ao+Pf	38.89	52.77	70.83	54.16
At+Pf	45.83	58.33	69.44	57.87
Cr+Pf	34.83	66.66	70.83	57.44
Control	13.89	16.66	20.83	17.13
Average	38.48	52.5	65.83	

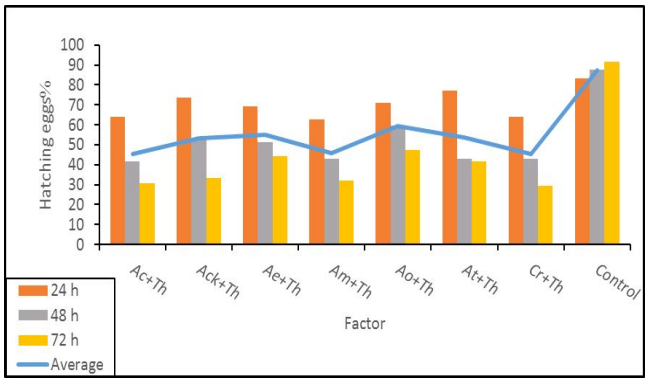

Fig. 3: Effect of NTF species in combination with *T. harzianum* filtrates on hatching eggs percentage

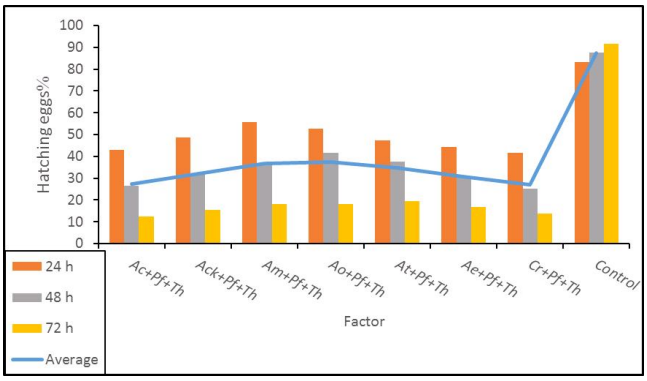

Fig. 4: Effect of combined filtrate of NTF species, *P. fluorescens* and *T. harzianum* on hatching eggs percentage

Table 3: Effect of NTF species in combination with *T. harzianum* filtrates on J2 mortality

Influencing factor	Time (h)			Average
	24	48	72	
Ac+Th	51.39	63.88	75	63.42
Ack+Th	61.11	59.72	73.61	64.81
Ae+Th	40.27	44.45	73.61	52.77
Am+Th	45.83	58.33	65.27	56.48
Ao+Th	34.7	55.55	72.22	54.16
At+Th	44.44	54.16	70.83	56.48
Cr+Th	40.28	63.88	72.22	58.79
Control	12.5	15.27	20.83	16.2
Average	41.32	51.91	65.45	

Table 4: Effect of combined filtrate of NTF species, *P. fluorescens* and *T.harzianum* on J2 mortality

Influencing factor	Time (h)			Average
	24	48	72	
Ac+Pf+Th	61.11	72.22	90.27	74.53
Ack+Pf+Th	62.5	68.05	86.11	72.22
Am+Pf+Th	50	63.89	81.94	65.28
Ao+Pf+Th	55.55	62.5	86.11	68.05
At+Pf+Th	54.16	65.28	81.94	67.13
Ae+Pf+Th	47.22	69.44	83.33	66.66
Cr+Pf+Th	63.88	73.61	87.5	75
Control	12.5	16.66	19.44	16.2
Average	50.87	61.46	77.08	

harzianum produced a significant reduction of hatching eggs and increasing of J2 mortality. The lowest hatching eggs percentage was 45.38% which observed when using either Ac+Th or Cr+Th filtrates, on the other hand, Ao+Th filtrates proved to be less efficient in reducing eggs hatching 59.26%. Moreover, hatching eggs percentage was 70.57% after 24 h then decreased to 43.75% after 72 h (Fig. 3). Table 3 explains that culture filtrates of Ack+Th revealed high J2 mortality of about 64.81%, followed by Ac+Th filtrates (63.42%), comparing with Ae+Th filtrates which was 52.77%. After 24 h of treatment, J2 mortality was 41.32%, then increased to 65.45% after 72 h.

The results showed that the combination of the three biological control agents was the most efficient in reducing the rate of eggs hatching and in increasing the J2 mortality. The lowest percentage of hatching eggs was 26.85% caused by combined filtrates Cr+Pf+Th, while the highest percentage was observed using Ao+Pf+Th filtrates (37.5%) (Fig. 4).

Table 4 indicates that the highest J2 mortality appeared when using Cr+Pf+Th filtrates (75%) followed by Ac+Pf+Th filtrates (74.53%), while Am+Pf+Th filtrates produced the lowest percentage of J2 mortality. Anyway, 24h gave low J2 mortality percentage reached to 50.87%, which was increased to 74.53% after 72h.

Discussion

The root-knot nematodes (*Meloidogyne* spp.) are sedentary endoparasites that are attacking a wide range of vegetables and other economically important agricultural (Singh and Mathur, 2010). It is considered the most damaging agricultural pests. Biological control of *Meloidogyne* spp. with antagonistic fungi and bacteria is a promising technique which can be incorporated in integrated nematode management. Therefore, using more than one BCA may give an effective control of *Meloidogyne* spp. (Askary, 2015).

Herrera-Estrella *et al.* (2016) showed that nematode trapping fungi effectively decrease the respective nematode population in laboratory and fields. Many studies indicated that *P. fluorescens* and *T. harzianum* reduced egg production in the root-knot nematode *Meloidogyne* spp. following soil treatments (Sharon *et al.,* 2001; Bagheri *et al.,* 2013).

Culture filtrates of NTF species, *P. fluorescens* and *T. harzianum* revealed a significant biocontrol activity against hatching eggs and J2 mortality of *Meloidagyne*

spp when applied separately, or in combination (NTF species with either *P. fluorescens* or *T. harzianum*), or in combination (NTF species with *P. fluorescens* and *T. harzianum*) and the later combination gave the best antagonistic effect. The maximum reduction of hatching eggs according to the above categorizes was appeared using *C. rosea* filtrate (59.73%), Ac+Th and Cr+Th filtrates (45.38% both) and Cr+Pf+Th filtrates equal to 26.85%.

Nourani *et al.* (2015) reported that *A.oligospora* and *A.conoides* have significant inhibitory activity against J2 of root-knot nematode (*M.incognita*) and some of them showed nematicidal effects (from 19 to 100% level of mortality). These results are in consistent with many studies that have indicated that *C. rosea* produces hydrolytic enzymes such as proteases, collagenase and chitinase which are involved in penetration of the cuticle of nematode and deterioration of the host cell as well as effects the second stage juveniles J2 (Hussain *et al.,* 2017). Blaxster *et al.,* (1998) confirmed that the J2 cuticle is composed mainly of proteins and Dackman *et al*., (1989) showed that nematophagous fungi produce proteinases which have effects on nematode eggs and J2.

Bagheri *et al.* (2013) mentioned that bacterial extracts of *P. fluorescens* inhibited the movement of second stage larvae of nematodes in water agar medium and affected the second stage larvae mortality rate. *Trichoderma* chitinolytic enzyme systems play an important role in egg-parasitism (Szabó *et al.*, 2012). *Arthrobotrys*, *C. rosea* and *Trichoderma* responsibility for the rapid J2 mortality may due to secretion of toxic metabolites that produced by these fungi (Migunova *et al.,* 2018). Moreover, Tranier *et al*. (2014) reported that the genera *Arthrobotrys, Trichoderma, Paecilomyces* and *Clonostachys* are used as potential and efficient biological agents against plant parasitic nematodes.

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