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Original article (Orijinal araştırma)

Effects of some *Metarhizium anisopliae* (Metschn.) Sorokin, 1883 (Hypocreales: Clavicipitaceae) isolates on root-knot nematodes under laboratory conditions[1]

Bazı *Metarhizium anisopliae* (Metschn.) Sorokin, 1883 (Hypocreales: Clavicipitaceae) izolatlarının laboratuvar koşullarında kök-ur nematodları üzerine etkileri

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Abstract

The aim of this study to isolate and diagnose entomopathogenic fungi obtained from soil samples taken from the plateaus and pastures of Sakarya Province in 2019, and to investigate the use of five isolates (S43/1, S43/2, S43/3, S42/1, S42/2) of the diagnosed as *Metarhizium anisopliae* (Metschn.) Sorokin, 1883 (Hypocreales: Clavicipitaceae) for control *Meloidogyne incognita* (Kofoid & White, 1919) and *Meloidogyne javanica* (Treub, 1885) (Tylenchida: Meloidogynidae). The study was conducted at the Laboratory of Nematology in Plant Protection Central Research Institute (Republic of Turkey Ministry of Agriculture and Forestry) in 2020. The experiments were performed under laboratory conditions. Counts of dead larvae were made 24, 48 and 72 h post application and mortality rates calculated. Isolate S43/1 was the most effective isolate for control *M. javanica* at 10^8 cfu/ml causing 98.5% mortality after 24 h and 100% after 48 h. Isolate S42/2 was the most effective against *M. incognita* at 10^8 cfu/ml and with 97.1% mortality after 24 h and 100% after 72 h.

Keywords: Biocontrol, *Meloidogyne incognita, Meloidogyne javanica*, *Metarhizium anisopliae*

Öz

Bu çalışmanın amacı, 2019 yılında Sakarya İli yayla ve meralarından alınan toprak örneklerinden elde edilen entomopatojen fungusların izolasyonunun, teşhisinin yapılması ve *Metarhizium anisopliae* (Metschn.) Sorokin, 1883 (Hypocreales: Clavicipitaceae) olarak teşhisi yapılan beş adet izolatın (S43/1, S43/2, S43/3, S42/1, S42/2), *Meloidogyne incognita* (Kofoid & White, 1919) ve *Meloidogyne javanica* (Treub, 1885) (Tylenchida: Meloidogynidae) ile mücadelede kullanım olanaklarının araştırılmasıdır. Denemeler 2020 yılında Türkiye Cumhuriyeti Tarım ve Orman Bakanlığı, Zirai Mücadele Merkez Araştırma Enstitüsü Müdürlüğü Nematoloji Laboratuvarı'nda laboratuvar koşullarında yürütülmüştür. Uygulamadan 24, 48 ve 72 saat sonra ölü larva sayımları yapılmış ve ölüm oranları hesaplanmıştır. S43/1 izolatı *M. javanica'*nın kontrolünde en etkili izolat olmuş, 10^8 cfu/ml dozunda 24 saat sonunda %98,5 ve 48 saat sonunda ise %100 ölüme neden olmuştur. S42/2 izolatı *M. incognita*'ya karşı en etkili izolat olmuş 24 saat sonunda %97.1 ve 72 saat sonunda %100 ölüme neden olmuştur.

Anahtar sözcükler: Biyolojik savaş, *Meloidogyne incognita, Meloidogyne javanica*, *Metarhizium anisopliae*

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Introduction

Root-knot nematodes, *Meloidogyne* spp. Goeldi, 1892 (Tylenchida: Heteroderidae) cause economic damage on many crops all over the world. Their characteristic symptoms occur on underground parts of plants. Infected roots swell at the point of infection and galls form. Several control methods are available for reducing root knot nematode populations. The most common and effective method used in their management is chemical control. However, due to the negative effects of the chemicals used on human, animal and environmental health, it has become necessary to develop new control strategies. One of the alternative control strategies is the use of biological control agents (Devi, 2018). Biological control of nematodes is one of the most important strategies in nematode control for sustainable agriculture (Mokhtari et al., 2009).

Certain soil-dwelling fungi are pathogenic on plant pests, including nematodes (Dijksterhuis et al., 1994). Some fungi are biological control agents and are important in the control of nematodes (Butt et al., 2001). Given that many fungi live within the rhizosphere along with nematodes and are in contact with them, they can be effective in reducing nematode populations in many soil and geographic contexts (Siddiqui & Mahmood, 1996). About 80 species of fungus belonging to more than 30 genera are effective against nematodes (Viaene & Abawi, 2000; Sun et al., 2006; Bakr et al., 2014). *Metarhizium anisopliae* (Metschn.) Sorokin, 1883 (Hypocreales: Clavicipitaceae) is an entomopathogenic fungus that occurs naturally in soil all over the world and causes disease in various insects and is widely used in biological control programs (Richards & Rogers, 1990; Driver et al., 2000; Liu et al., 2007; Hoe et al., 2009; Abdollahi, 2018). *Metarhizium anisopliae*, the agent of green muscardine disease of insects, is the most important entomopathogenic fungus (Driver et al., 2000). This is a facultative parasite which can infect a range of insects and is a well-studied for microbial control of insect pests (Liu et al., 2007; Hoe et al., 2009).

The exact mode of action of *M. anisopliae* on nematodes is still unknown but it is likely to be similar to other fungi with sticky spores or conidia. The conidia germinate, parasitize and kill the nematode, by direct penetration and producing the infective hyphae inside the nematode body. This fungus produces some cyclic peptides, destruxins which may be important in its pathogenicity (Kershaw et al., 1999; Hsiao & Ko, 2001). Prior to infection of the host, the fungus produces destruxins A and B that can kill the host (Roberts, 1966).

There are only a few reports of the impact of *M. anisopliae* on nematodes. The effectiveness of *M. anisopliae* on nematodes has been demonstrated in studies conducted with *Rotylenchulus reniformis* Linford & Oliveira, 1940 (Tylenchida: Hoplolaimidae) (Tribhuvaneshwar et al., 2008), *Heterodera avenae* Wollenweber, 1924 (Tylenchida: Heteroderidae) (Ghayedi & Abdollahi, 2013) and several root-knot nematode species (Jahanbazian et al., 2014).

This study aimed to isolate and identify entomopathogenic fungi from 50 soil samples taken from the meadows and pasture areas of Sakarya Province, Turkey. The activity of five isolates of *M. anisopliae* against *Meloidogyne incognita* (Kofoid & White, 1919) and *Meloidogyne javanica* (Treub, 1885) (Tylenchida: Meloidogynidae) was investigated under laboratory conditions.

Materials and Methods

Soil samples were collected from the plateaus and pastures of Sakarya Province in 2019. From these five isolates (S43-1, S43-2, S43-3, S42-1, S42-2) of *M. anisopliae* were tested against *M. incognita* and *M. javanica*. The study was conducted at the Plant Protection Central Research Institute of the Laboratory of Nematology (Republic of Turkey Ministry of Agriculture and Forestry) in 2020.

Collection of soil samples

In July 2019, 50 soil samples were collected from meadows and pastures of Sakarya Province. Samples were taken from various locations to depths of 15-20 cm with the locations were chosen arbitrarily (Mracek & Becvar, 2000). Five to six subsamples were taken 5-6 m apart at each location and mixed thoroughly. About 1 kg of soil was collected at each location. The coordinates of the samples were recorded, each soil sample was labeled with a code number and brought to the laboratory in plastic bags.

Production of *Galleria mellonella* larvae

The final instar of the larvae of *Galleria mellonella* (L., 1758) (Lepidoptera: Pyralidae), also known as the large wax moth, were used in the isolation of entomopathogenic fungi from the soil samples. *Galleria mellonella* larvae were grown in artificial medium consisting of 45 g beeswax, 90 g granulated yeast, 307 g maize flour, 225 g honey mixture in glass jars at 27 ± 1ºC (Kaya & Stock, 1997). A proportion of the reared larvae were retained for pupal and adult development to ensure the continuation of the culture.

Entomopathogenic fungus isolation from soil

Entomopathogenic fungus isolation from the soil was performed using *G. mellonella* as bait (Zimmermann, 1986; Griffin et al., 2000). Prior to the isolation, the final instar of *G. mellonella* were submerged in 50 ± 3ºC water for 5 s and then transferred to a cold-water bath for 3 s to prevent spinning of web when they were released into the soil samples (Woodring & Kaya, 1988). Soil samples (300 g) were dispensed into 500 ml plastic containers and moistened with sterile water; 10 larvae of the same size were added to these containers and kept in an incubator at 22-25ºC in the dark. Samples were checked every 3 d for 10-15 d and dead larvae were removed. This was repeated three times for each sample.

Dead larvae collected from the soil were placed in 3% sodium hypochlorite for 3 min, then rinsed twice with sterile distilled water and placed in 9-cm Petri dishes of potato dextrose agar (PDA; Merck, Darmstadt, Germany). Agar plates were incubated at 24 ± 1ºC, with a 12:12 h L:D photoperiod for one week. Fungal colonies on the PDA medium were subcultured on PDA into 6-cm Petri dished, and a single spore isolation was made from the fungus colony that developed and stored in 20% glycerol at -20ºC in inclined tubes with PDA at 4ºC.

Molecular identification of entomopathogenic fungi

DNA extraction and PCR conditions

Total DNA was extracted according to DArT DNA isolation method (http://www.diversityarrays.com). Fungal mycelia were scraped from the surface of 15-d cultures grown on PDA and placed into 1.5 ml Eppendorf tubes. The samples were homogenized in 650 µl buffer solution [125 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0, 0.8 M NaCl, 1% CTAB, 1% sarcosyl and 2% PVP-40 (K29-32), 0.5% sodium disulfite] and then incubated at 65ºC for 1 h. The suspension was mixed with one volume of chloroform: isoamylalcohol (24:1) and centrifuged for 20 min at 13,000 g. DNA was precipitated with the same volume of ice-cold isopropanol. The pellet was washed with 70% cold ethanol, air-dried and suspended in 50 µl of sterile water. DNA concentrations were determined spectrophotometrically and finally diluted to 20 ng/µl.

The nuclear internal transcribed-spacer region (ITS1-5.8S-ITS2) of the isolates obtained was amplified using primer pairs ITS1 (5'- TCC GTA GGT GAA CCT GCGG -'3) and ITS4 (5'- TCC TCC GCT TAT TGA TATGC -'3) described by White et al. (1990). PCR reactions were performed in 50 µl containing 5 µl 10× reaction buffer, 0.4 µM of each primer, 0.2 mM of dNTPs, 1.5 mM of $MgCl_2$, 1.5 U *Taq* DNA polymerase (Thermo Fischer Scientific, Waltham, MA, USA) and 20 ng DNA. PCR cycling conditions were as follows: 4 min at 94ºC; 45 s at 94ºC, 45 s at 56ºC, 1 min at 72ºC for 35 cycles; and 10 min at 72°C. Amplification products were checked on 1.2% agarose gel stained with ethidium bromide. PCR products were sequenced in both directions using the same primers by Macrogen (Seoul, Korea).

Sequence data was optimized with the software package Seqman (DNAStar Inc., Madison, WI, USA). The alignments of the resulting sequences and the reference isolates from Genbank were performed by MEGA7 (Kumar et al., 2016). The tree showing genetic relationships among the isolates was constructed by the neighbor-joining method, Kimura 2-parameter distances and 1000 bootstrap values.

Generation of second stage juvenile of root-knot nematode species

Pure cultures of *M. incognita* and *M. javanica*, previously identified and stored in the Nematology Laboratory of the Directorate of Plant Protection Central Research Institute (Ankara), were used in this study. Serial cultures of susceptible tomato plants (Tueza F1) were used to culture these nematodes. The infected roots were washed carefully under tap water and egg masses were collected from these roots under the microscope and left to hatch at room temperature. The juveniles that hatched in the first 24 h were discarded and subsequent hatching were monitored and juveniles collected daily. The suspension with nematodes obtained was adjusted to 100 juveniles/ml.

Preparation of entomopathogenic fungi

Metarhizium anisopliae isolates were inoculated on Sabouraud dextrose agar in 9-cm Petri dishes and incubated at 25 ± 1ºC in the dark for 15 d. After the incubation, 2 ml of sterile distilled water with 0.03% Tween 80 was added to each Petri dish to suspend the spores by scraping with a glass rod. The spore suspensions were filtered through a four-layer cheesecloth and separated from mycelium for 3 min. Spore suspensions were homogenized with a vortex mixer and diluted to 1×10^6, 10^7 and 10^8 spores/ml with a Neubauer hemocytometer (Saruhan et al., 2015). Spore germination rates were determined before using the isolates. One hundred µl of the suspension diluted 1×10^4 was spread on PDA in three Petri dishes and incubated in the dark at 25 ± 1ºC for 24 h. In each Petri dish, 100 spores were examined and the germination rate determined as ≥90% (Güven et al., 2015).

Application of entomopathogenic fungi to root knot nematode species

The experiments were conducted in the laboratory in 24-well culture plates. Second stage juveniles of each nematode species were transferred to separate wells in the culture plates with a dropper at 100 juveniles/ml. Then, 1×10^6, 10^7 and 10^8 spores/ml suspensions of *M. anisopliae* isolates were added to the wells. Each test was done with three replicates and two repeats. Viability rates of nematodes were determined after 24, 48 and 72 h, and nematode mortality rates calculated (Abbasi et al., 2018). Dead juveniles were examined under light microscope to determine if they had been killed by fungal infection. Only pure water was used in the control.

Statistical analysis

The data were evaluated using Duncan multiple comparison test in SPSS program. Square root transformation was applied to non-normally distributed data, followed by ANOVA Duncan test.

Results and Discussion

Metarhizium-like fungi were isolated from 50 soil samples collected from different meadow and pastures in Sakarya Province. Five isolates from samples S42 and S43 were identified as *M. anisopliae* based on morphological characteristics (Humber, 1997) (Figure 1) and confirmed by DNA sequence analysis of ribosomal DNA-ITS region. PCR amplification with the primers ITS1 and ITS4 yielded a single DNA fragment of about 580 bp for all isolates. BLASTn search of their sequences showed high level of genetic similarity with DNA sequences of *M. anisopliae* available in GenBank. The resulting sequences were deposited in the GenBank database (Accessions MW073447 to MW073451). Neighbor-joining tree derived from DNA sequences of *M. anisopliae* and closely related fungi corresponded to the morphological classification (Figure 2). The sequence alignments revealed high level of identity between the isolates of the same species. *Metarhizium anisopliae* isolates were placed in a unique group different from other *Metarhizium* spp.

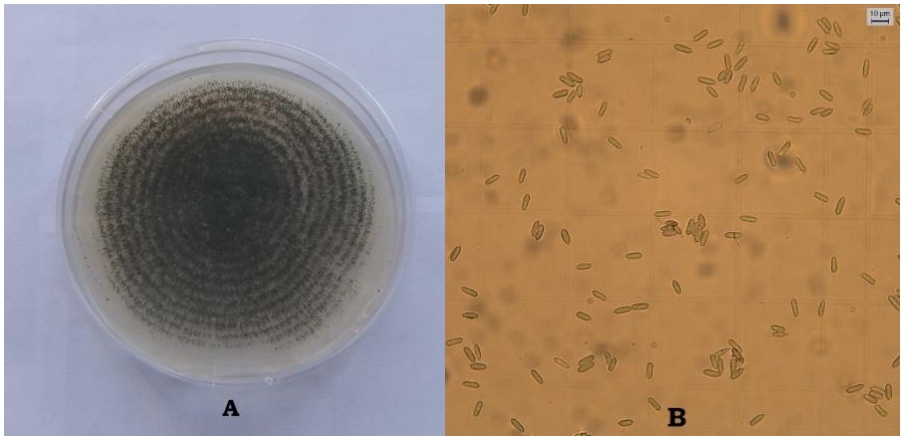

Figure 1. a) 15-day colony of *Metarhizium anisopliae* on PDA, and b) conidia at 40x magnification.

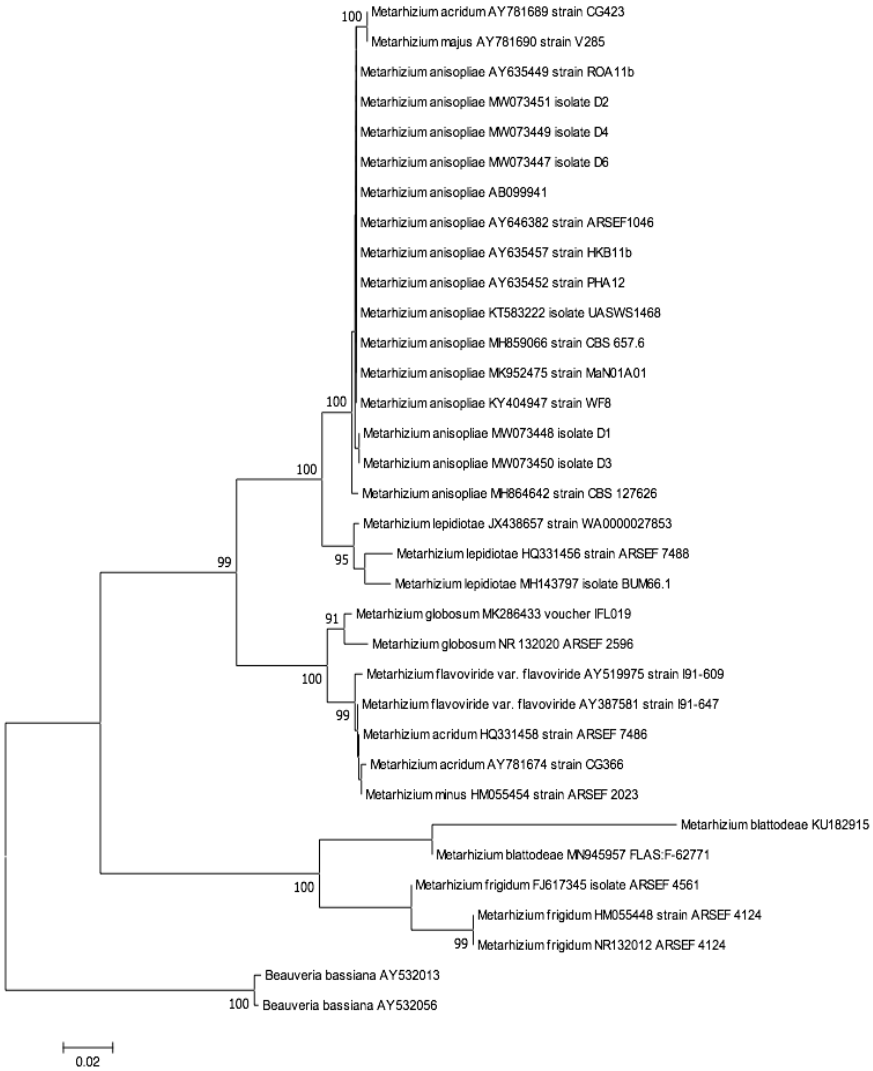

Figure 2. Phylogenetic analysis of *Metarhizium* spp. isolates based on ITS rDNA sequences. The bootstrap values were obtained from 1000 replications and the values greater than 90% are shown in the dendrogram. The ITS sequences of *Beauveria bassiana* were used as an outgroup.

The efficacy of five *M. anisopliae* isolates against both *M. incognita* and *M. javanica* increased as the dose increased (Tables 1 & 2).

Table 1. Mortality (%) of *Meloidogyne javanica* larvae caused by different *Metarhizium anisopliae* isolates after 24, 48 and 72 h

Doses	*Metarhizium anisopliae* isolate									
	S43/1		S43/2		S43/3		S42/1		S42/2	
24 h										
10^{-6}	69.9 ± 4.74	d	17.9 ± 1.93	f	17.9 ± 1.93	f	16.5 ± 1.46	f	15.7 ± 1.73	f
10^{-7}	91.9 ± 0.50	ab	31.0 ± 3.23	e	31.0 ± 3.23	e	77.7 ± 4.40	c	78.5 ± 1.93	c
10^{-8}	98.5 ± 0.44	a	88.5 ± 2.48	b	88.5 ± 2.48	b	95.2 ± 0.74	ab	91.9 ± 1.03	ab
48 h										
10^{-6}	85.3 ± 1.28	ef	27.7 ± 2.99	h	58.9 ± 7.26	g	17.3 ± 1.43	i	19.4 ± 2.24	i
10^{-7}	93.9 ± 0.64	abcd	91,4 ± 1,43	bcde	86.7 ± 2.09	def	89.3 ± 2.23	cde	81.1 ± 2.01	f
10^{-8}	100.0 ± 0.00	a	95.8 ± 1.31	abc	98.3 ± 0.95	ab	97.7 ± 0.50	ab	94.6 ± 1.01	abc
72 h										
10^{-6}	86.8 ± 1.45	c	70.4 ± 1.12	e	75.2 ± 2.17	d	24.6 ± 2.54	f	26,1 ± 3,67	f
10^{-7}	95.3 ± 0.44	ab	95.1 ± 1.39	ab	91.8 ± 1.98	b	96.8 ± 0.61	a	86.8 ± 1.44	c
10^{-8}	100.0 ± 0.00	a	99.8 ± 0.18	a	99.7 ± 0.22	a	99.1 ± 0.33	a	100.0 ± 0.00	a

$F_{1,47}$h = 224, 212 and 446 for 24, 48 and 72 h, respectively.

For *M. javanica,* isolate S43/1 was 98.5% effective at 10^8 cfu/ml after 24 h and 100% after 48 h. After 72 h, isolates S43/1 and S42/2 were also 100% effective at 10^8 cfu/ml. isolates S43/2, S43/3 and S42/1 were 99.8, 99.7, 99.1% effective, respectively.

Table 2. Mortality (%) of *Meloidogyne incognita* larvae caused by different *Metarhizium anisopliae* isolates after 24, 48, 72 h

Doses	*Metarhizium anisopliae* isolate									
	S43/1		S43/2		S43/3		S42/1		S42/2	
24 h										
10^{-6}	5.1 ± 0.53	d	3.8 ± 0.83	e	1.54 ± 0.44	f	-0.4 ± 0.34	g	-0.5 ± 0.35	g
10^{-7}	5.8 ± 0.50	d	5.5 ± 0.79	d	85.5 ± 2.12	ab	71.9 ± 2.77	c	74.7 ± 1.40	bc
10^{-8}	88.9 ± 1.97	a	95.7 ± 0.48	a	95.4 ± 0.79	a	94.7 ± 0.62	a	97.1 ± 0.41	a
48 h										
10^{-6}	5.3 ± 0.62	d	3.3 ± 0.78	e	1.2 ± 0.49	f	-0.7 ± 0.58	h	-0.5 ± 0.44	gh
10^{-7}	78.9 ± 2.29	c	5.0 ± 0.91	d	89.7 ± 1.49	abc	82.3 ± 1.58	abc	80.9 ± 1.55	bc
10^{-8}	96.4 ± 0.57	ab	98.6 ± 0.51	ab	98.5 ± 0.44	ab	96.7 ± 0.32	ab	99.7 ± 0.22	a
72 h										
10^{-6}	11.6 ± 2.28	c	75.3 ± 1.38	b	1.2 ± 0.49	d	-0.9 ± 0.68	f	0.7 ± 0.58	de
10^{-7}	93.1 ± 1.05	a	86.5 ± 0.56	ab	94.6 ± 1.46	a	86.7 ± 1.04	ab	96.0 ± 0.48	a
10^{-8}	97.6 ± 0.63	a	99.3 ± 0.34	a	99.0 ± 0.38	a	98.6 ± 0.35	a	100.0 ± 0.00	a

$F_{1,47}$ = 788, 558, and 528 for 24, 48 and 72 h, respectively.

For *M. incognita*, isolate S42/2 was 97.1% effective at 10^8 cfu/ml after 24 h, whereas isolates S43/2 and S43/3 were 95.7 and 95.4% effective, respectively. Isolate S42/2 was also effective in counts made after 3 d and it was 100% effective after 72 h. This was followed by S42/1, S43/3, S42/1 and S4 /1 (99.31%, 98.97, 98.61, 97.58). Some species of *Metarhizium* have the ability to colonize roots (Bruck, 2005). Some isolates of *M. anisopliae* have endophytic behavior (Leger, 2008). The fungus produces sticky conidia that attach to nematode cuticles (Ghayedi & Abdollahi, 2013). The conidia germinate, parasitize and kill the nematode, by direct penetration producing infective hyphae inside the nematode body. The fungus produces some cyclopeptides and destruxins which are important in its pathogenicity (Kershaw et al., 1999).

In previous studies in Turkey, *M. anisopliae* isolates were obtained from grass, *Lolium* spp. Lam. (Poales: Poaceae) by Sevim et al. (2012) in the Eastern Black Sea Region, Er (2013) in pistachio, *Pistacia vera* L. (Sapindales: Anacardiaceae) orchards Gaziantep, Gürlek et al. (2018) in the walnut, *Juglans* spp.

(L.) (Fagales: Juglandaceae) orchards in Kırşehir; Tuncer et al. (2018) in nut weevil, *Curculio nucum* (L., 1758) (Coleoptera: Curculionidae), and Keskin et al. (2019) in Düzce and their effectiveness against various pests investigated. However, no study has been conducted in Turkey on the efficacy of *M. anisopliae* on root-knot nematodes.

In the present study, it was observed that fungal efficacy increased with increased concentrations and the lowest effect was recorded at 10^6 cfu/ml for all isolates. These results are consistent with other studies. A study assessing the efficacy of *M. anisopliae* and *Trichoderma harzianum* Rifai on *M. javanica* under laboratory and greenhouse conditions, found that these fungi had no antagonistic effect on each other and that both fungi were effective against root knot nematodes, but there was no significant benefit from combined application (Abdollahi, 2015).

Abdollahi (2018) determined the effectiveness of *M. anisopliae* IMI 330189 and different oak, *Quercus* spp. (Fagales: Fagaceae) tree debris on *M. javanica* in tomato and demonstrated that the combination of *M. anisopliae* and oak tree debris caused a reduction of over 90% in nematode development with 100 and 150 g/kg soil. Although, *M. anisopliae* caused a 76% decrease in the number of galls formed in the roots compared to the control, its combination with oak debris caused a decrease of 86% and did not reduce the effect of entomopathogenic fungi.

In another study (Youssef et al., 2020), the effect of different concentrations of *Beauveria bassiana* (Bals.-Criv.) Vuill*., M. anisopliae* and *Paecilomyces lilacinum* (Thom) Samson spores and filtrates on *M. incognita* egg hatch and juvenile mortality in cowpea was investigated in climatic chamber. The highest mortality with the standard dilution occurred with *P. lilacinus* at 84.5%, followed by *B. bassiana* and *M. anisopliae* at 81.1% and 78.5%, respectively. With 10^8 cfu/ml^{1}n, *P. lilacinus* at 85.3% mortality was followed by *M. anisopliae* at 83.6%. All three fungi were effective against *M. incognita* and could be used in biological control of the agent.

In a study of the nematicidal activity of *B. bassiana* against *Meloidogyne hapla* Chitwood, 1949 was evaluated under greenhouse conditions (Liu et al., 2008), it was observed that hatching and mortality rates were directly proportional to the density of *B. bassiana*. It has been observed that *B. bassiana* significantly reduces larval density, egg sac and gall formation in the soil and root.

In the present study, five *M. anisopliae* isolates from Turkey gave over 95% mortality after 72 h at 10^8 cfu/ml. Therefore, based on this study conducted under laboratory conditions, it is considered that detailed semi-field and field trials would be justified. If efficacy is demonstrated in the semi-field and field trials these isolates could be used to control root knot nematodes (*Meloidogyne* spp.) in integrated pest control programs.

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