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Entomopathogenicity of *Metarhizium anisopliae* and some fungi toward the filbert aphid, *Myzocallis coryli* Goetze (Hemiptera: Aphididae)

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Abstract
The Filbert Aphid is one of the pests of hazelnut growing area of Turkey. This study was carried out all fungal isolates caused significant mortality against third instar nymphs of filbert aphid after 3, 6 and 10 days of conidial treatments at 18, 22 and 25 °C temperatures under laboratory conditions. The results showed that third instar nymphs of *M. coryli* were more susceptible to the entomopathogenic fungi at 25 °C than 18 °C. *M. anisopliae* was highly and statically equally effective against nymphs of *M. coryli* at 18, 22 and 25 °C (80.8%, 93.8%, and 100%) after 10 days of conidial treatment. The other isolates; TR-05 and TR-78.07 weren't as effective as *M. anisopliae* on nymphs of *M. coryli* at 25 °C (67.8 and 62.6%). This study showed that isolate of *M. anisopliae* has virulent and highly potential for biological control on nymphs of *Myzocallis coryli.*

Keywords: *Myzocallis coryli, Metarhizium anisopliae*, entomopathogenic fungi, biological control

1. Introduction
Aphids are serious pests of cultivated crops all over the world and their infestations can lead to severe economic losses as a result of crop yield reduction. Direct damage occurs due to sucking plant sap from succulent parts of plant and heavy infestations results in leaf fall, lack of maturity of fruits and death of plant [15]. The hazelnut pest *Myzocallis coryli* (Goeze) is a European aphid that has expanded into the rest of the world and is now found on every continent. Its main host trees and shrubs are of the genus Corylus (*C. avellana, C. colurna, C. heterophylla and C. máxima*) [6].

Filbert aphid was historically a key pest in hazelnut orchards managed with several sprays of organophosphates each season in order to prevent crop losses. This aphids cause both direct and indirect damage due to the fact that they attack both leaves and husks. Excessive feeding on leaves can cause early leaf drop and high aphid populations on husks may result in the drying of husks resulting in low quality nuts [14, 35].

Most of the hazelnut farmers use pesticides and spray 1-2 times every year for to protect their products from aphids and other pest in Turkey [46]. Currently used chemical pesticides are usually relatively cheap and efficient, supply chains exist and growers are equipped to apply those [27]. But chemical pesticides and other highly effective crop protection methods often promote the development of pest resistance because they impose a high selection pressure on the pest populations [28, 21, 34].

In addition exposure to pesticides is one of the most important occupational risks among farmers in developing countries [49, 22, 8]. National and EU legislative directives have been imposed to limit pesticides and thus their negative impacts on the environment and human health [42, 25, 1]. Several restrictions need to be overcome for alternative pest control methods to be adopted [27]. Biological control is an alternative control method for insect pests. Biological control regarded as sustainable methods in agriculture systems due to its natural origin and low environmental side effects. In biological control parasitoids, predatory arthropods and other invertebrates, competing microorganisms and pathogens used [40].

Entomopathogenic bacteria, viruses, fungi and nematodes have been reported as plant protection agents against several insects [38]. Entomopathogenic fungi are generally considered to be safe in terms of low risks as compared to chemical pesticides. New areas for use of these fungal biocontrol agents include their use in close proximity to foods and feed, or even applied directly to stored grains as well as to other food commodities [9, 10].

The entomopathogenic fungus *Metarhizium anisopliae* can infect 200 species from more than 50 insect families [37], and is used globally as a biological control agent for many insect pests. Its advantages over chemical pesticides include high insect specificity, low toxicity to other organisms and low environmental impact [29]. In addition, due to its multiple development processes of infection, insect resistance to *M. anisopliae* is less likely or develops more slowly than resistance to chemical pesticides [17]. The aim of this study was to investigate the pathogenicity of *M. anisopliae* and some entomopathogenic fungi in controlling Filbert Aphid (*Myzocallis coryli* Goetze).

2. Materials and Methods

2.1 Isolation of *M. anisopliae* from insect

The *Metarhizium anisopliae* were isolated from infected insects [*Xylosandrus germanus* (Coleoptera: Curculionidae: Scolytinae)] in hazelnuts orchards in the provinces of Samsun, Turkey. The insects were surface disinfected with 5% sodium hypochlorite and placed in an environmental chamber on a water agar medium amended with antibacterial agents, on moistened filter paper in a sealed container and incubated at 25±1 ºC for fifteen days. The insects with hyphae were then transferred to selective medium for the isolation of *M. anisopliae*. The fungus was then grown on Potato dextrose agar (Hi-Media) fortified with 1% yeast extract at 25±1 ºC in dark. Single-spore isolates were obtained by serial dilution [11] and identified as *M. anisopliae.*

2.2 Isolation of the 2 other fungal cultures

Fungal cultures were isolated from infected *Palomena prasina* (Heteroptera: Pentatomidae) and *Hyphantria cunea* (Lepidoptera: Arctiidae) in hazelnuts orchards in the provinces of Düzce and Samsun, Turkey. Single-spore isolates were obtained by serial dilution [11] and identified as *Lecanicillium muscarium* (isolate TR-05 from *P. prasina*) and *Isaria fumosorosea* (isolate TR-78.07 from *H. cunea*). Isolates were maintained in tubes containing 6.5% Sabouraud dextrose agar (SDA) (Merck Ltd., Darmstadt, Germany) and deposited in the fungal culture collection of the Mycology Laboratory at the Ondokuz Mayis University Faculty of Agriculture's Department of Plant Protection in Samsun, Turkey and in the USDA-ARS Entomopathogenic Fungal Culture Collection in Ithaca, NY (ARSEF 11731 and 12177 respectively) [39].

2.3 Conidial germination assessment

The viability of conidia of *M. anisopliae* and the 2 other isolates (TR-05 and TR-78.07) were evaluated using a method modified from [25]. A conidial suspension was adjusted to 1×10^4 conidia/mL, and 0.2 mL was sprayed onto 9-cm-dia. Petri plates containing potato dextrose agar (PDA) (Oxoid Ltd, Basingstoke, UK). Petri plates were maintained at 25±1 °C. After 24 h of incubation, percentages of germinated conidia were counted using an Olympus CX-31 compound microscope at 400x magnification. Conidia were regarded as germinated when they produced a germ tube at least half of the conidial length. Germination ratios for each fungus were calculated after examining a minimum of 200 conidia from each of 3 replicate plates [39].

2.4 Inoculum of entomopathogen isolates

Isolate of *M. anisopliae* and the 2 other isolates TR-05 and TR-78.07) were grown on SDA at 25±1 °C for 15 days. Conidia were harvested with sterile distilled water containing 0.03% Tween 80. Mycelia were removed by filtering conidia suspensions through 4 layers of sterile cheesecloth. Conidia were counted under a compound microscope using a Neubauer hemocytometer to calibrate a suspension of 1×10^8 conidia/mL for each isolate [39].

2.5 Commercial products

The effects of *M. anisopliae* were compared with those of commercially available biocontrol products [Nostalgist BL (SL; *Beauveria bassiana*, Bb-1 %1.5, $1x10^8$ kob/ml min.) and Nibortem (SL; *Verticillium lecanii* V1-1 %1.5, $1x10^8$ kob/ml min.)] at a dosage: 250 mL Nostalgist BL /100 L water and 250 mL Nibortem /100 L water. The commercial products were diluted to recommended rates for used this study.

2.6 Insect rearing

The female adults of filbert aphid were collected from hazelnut orchards in Samsun, Turkey, during early June of 2015. They were reared as a group of 10 female adults separately on hazelnut leaves in 13 cm diameter dishes and established same groups with 1000 females. Females get birth and then nymphs collected and transferred to new 9 cm diameter dishes and they reared as a group of 10 nymphs separately on hazelnut leaves. Nymphs were in growth chamber (18 ± 1 °C, 22 ± 1 °C, 25 ± 1 °C ; 75 ± 5% R.H; 16:8 h L:D) and they stayed in there to turn third instar nymphs. One day old nymphs of third instars were used for the virulence tests.

2.7 Bioassay

Third instar nymphs of *Myzocallis coryli* were placed on hazelnut leaves in 9 cm diameter dishes containing sterile water-soaked blotters (10 nymphs per dishes). Conidial suspensions of *M. anisopliae* and the 2 other entomopathogenic fungi (TR 05 and TR 78.07) and the 2 other products (Nibortem and Nostalgist BL) were applied to the third instar nymphs of *M. coryli* (2 mL per dishes) using a Potter spray tower (Burkard, Rickmansworth, Hertz UK). Control units were treated with sterile distilled water (2 mL). Each of dishes was loosely capped to prevent escape after applications. Dishes were incubated at 18 ± 1 °C, 22 ± 1 °C and 25 ± 1 °C at 75±5% and 16:8h L:D photoperiod for 10 days. All dishes were inspected daily. Dead nymphs of *M. coryli* were counted and removed into empty dishes. Mortality of nymphs was recorded on 1-10 days after treatment. The experiment was repeated 10 replicates per treatment.

2.8 Statistical analysis

The mortality percentage of nymphs for each isolates and products were analyzed using one way ANOVA (SPSS 21 for Windows); means were separated by Duncan's mean separation test. Mortality was considered significantly different at $P<0.001$.

3. Results and Discussion

Dose-response relationship was determined for *M. anisopliae* and the other entomopathogenic fungi applied to the third instar nymphs of *M. coryli* at 3 different temperatures in laboratory conditions by using spraying method. The accumulated mortality recorded during 1-10 days and showed that all isolates were found effective against on nymph at different rates (Figure 1, 2, 3). According to our study,

significantly different effects on mortality were observed among different isolates and commercial products at 3 different temperatures (p <0.001). Nymphs in control units survived to finish of experiments without any mortality. All living nymphs in all applications and control units were fed with hazelnut leaves in dishes. They transformed into adult after 10-12 days of application without any mortality.

3.1 Three days after application

M. anisopliae was the most efficacious in controlling third instar nymphs of *M. coryli* at 18, 22 and 25 °C temperatures (20.8%, 30.2%, and 38.8%). The other isolates; TR-05 (*L. muscarium*) and TR-78.07 (*I. fumosorosea*) weren't so effective on nymphs of *M. coryli* at 18 °C (12.0% and 10.0%), 22 °C (21.8% and 14.2%) and 25 °C (24.0% and 17.0%) temperatures. The commercial biocontrol products (Nibortem and Nostalgist BL) were less pathogenic on nymphs of *M. coryli* at 3 different temperatures and mean mortality ranged from 2.2 to 6.2% (Table 1).

3.2 Six days after application

All fungal isolates caused significant mortalities after 6 days of conidial treatments. *M. anisopliae* was equally effective against nymphs of *M. coryli* at 18, 22 and 25 °C (51.8%, 70.6%, and 82.0%). The other isolates; TR-05 (*L. muscarium*) and TR-78.07 (*I. fumosorosea*) weren't so effective on nymphs of *M. coryli* at 18 °C (28.6% and 26.2%), 22 °C (42.4% and 40.8%) and 25 °C (49.2% and 40.6%) temperatures. The commercial biocontrol products (Nibortem and Nostalgist BL) were least pathogenic on nymphs of *M. coryli* at 3 different temperatures and mean mortality ranged from 7.2 to 20.8% (Table 2).

3.3 Ten days after application

All fungal isolates caused highly significant mortalities after 10 days of conidial treatments. *M. anisopliae* was highly and statically equally effective against nymphs of *M. coryli* at 18, 22 and 25 °C (80.8%, 93.8%, and 100%). The other isolates; TR-05 (*L. muscarium*) and TR-78.07 (*I. fumosorosea*) weren't so effective on nymphs of *M. coryli* at 18 °C (49.0% and 47.4%), 22 °C (58.8% and 56.4%) and 25 °C (67.8% and 62.6%) temperatures. The commercial biocontrol products (Nibortem and Nostalgist BL) were least pathogenic on nymphs of *M. coryli* at 3 different temperatures and mean mortality ranged from 11.6 to 28.6% (Table 3).

Table 1: Mortality percentages of nymphs of *M. coryli* at 3 different temperatures after 3 days of application

Isolates and commercial products	Mortality percentage of nymphs after 3 days of application						
	18 °C		22 °C		25 °C		P*
M. anisopliae	20.8 ± 0.97	a*C**	30.2 ± 1.77	aB	38.8 ± 1.32	aA	<0.001
TR-05	12.0 ± 1.05	bB	21.8 ± 1.43	bA	24.0 ± 2.26	bA	0.001
TR-78.07	10.0 ± 0.63	bB	14.2 ± 0.73	cA	17.0 ± 1.26	cA	0.001
Nibortem	3.8 ± 0.97	cB	3.2 ± 0.58	dB	6.2 ± 0.58	dA	0.032
Nostalgist BL	2.2 ± 0.37	cB	2.4 ± 0.24	dB	4.8 ± 0.49	dA	0.001
P*	<0.001		<0.001		<0.001		

*The small letters within columns indicates significant differences between means (isolates)
**The capital letters within rows indicates significant differences between means (temperatures)

Table 2: Mortality percentages of nymphs of *M. coryli* at 3 different temperatures after 6 days of application

Isolates and commercial products	Mortality percentage of nymphs after 6 days of application						
	18 °C		22 °C		25 °C		P*
M. anisopliae	51.8 ± 0.86	a*C**	70.6 ± 1.44	aB	82.0 ± 1.76	aA	<0.001
TR-05	28.6 ± 1.21	bC	42.4 ± 1.36	bB	49.2 ± 1.24	bA	<0.001
TR-78.07	26.2 ± 0.73	bB	40.8 ± 0.92	bA	40.6 ± 1.57	cA	<0.001
Nibortem	11.0 ± 1.18	cB	11.2 ± 0.58	cB	20.8 ± 0.97	dA	<0.001
Nostalgist BL	7.2 ± 0.73	dB	10.2 ± 1.16	cB	18.4 ± 1.17	dA	<0.001
P*	<0.001		<0.001		<0.001		

Table 3: Mortality percentages of nymphs of *M. coryli* at 3 different temperatures after 10 days of application

Isolates and commercial products	Mortality percentage of nymphs after 10 days of application						
	18 °C		22 °C		25 °C		P*
M. anisopliae	80.8 ± 1.93	a*C**	93.8 ± 0.86	aB	100.0 ± 0.001	aA	<0.001
TR-05	49.0 ± 1.11	bC	58.8 ± 0.81	bB	67.8 ± 1.21	bA	<0.001
TR-78.07	47.4 ± 1.17	bC	56.4 ± 2.06	bB	62.6 ± 2.14	cA	<0.001
Nibortem	16.8 ± 1.02	cC	21.2 ± 1.32	cB	28.6 ± 1.08	dA	<0.001
Nostalgist BL	11.6 ± 0.68	dC	18.6 ± 1.29	cB	24.8 ± 1.24	dA	<0.001
P*	<0.001		<0.001		<0.001		

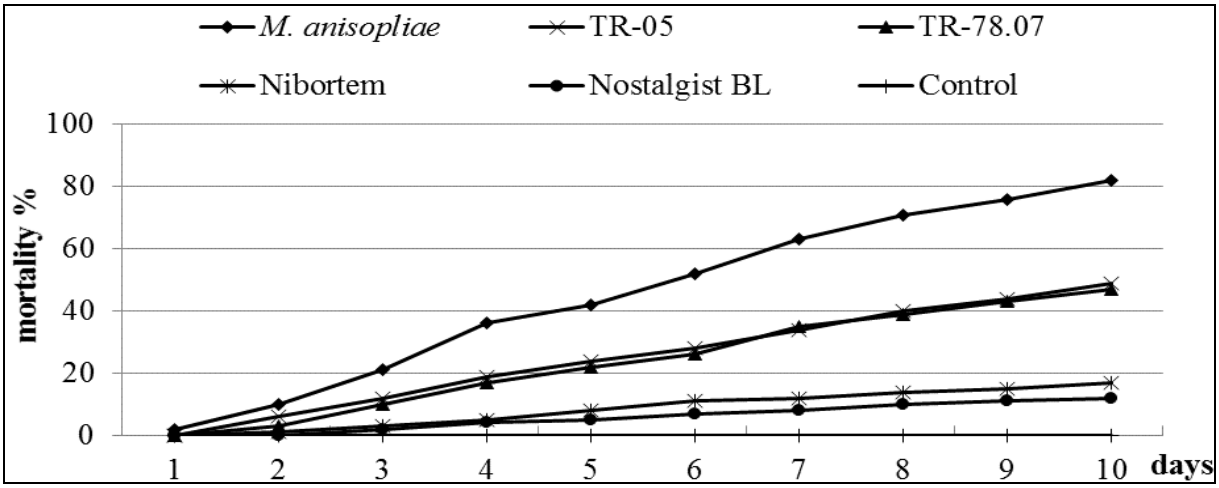

Fig 1: Cumulative mortality percentages of nymphs of *M. coryli* at 18 °C

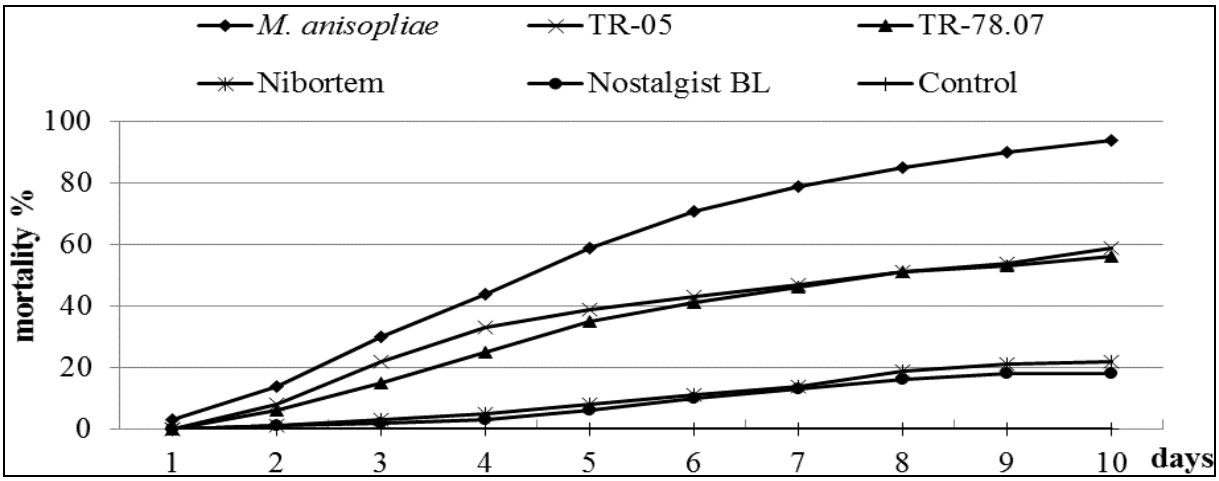

Fig 2: Cumulative mortality percentages of nymphs of *M. coryli* at 22 °C

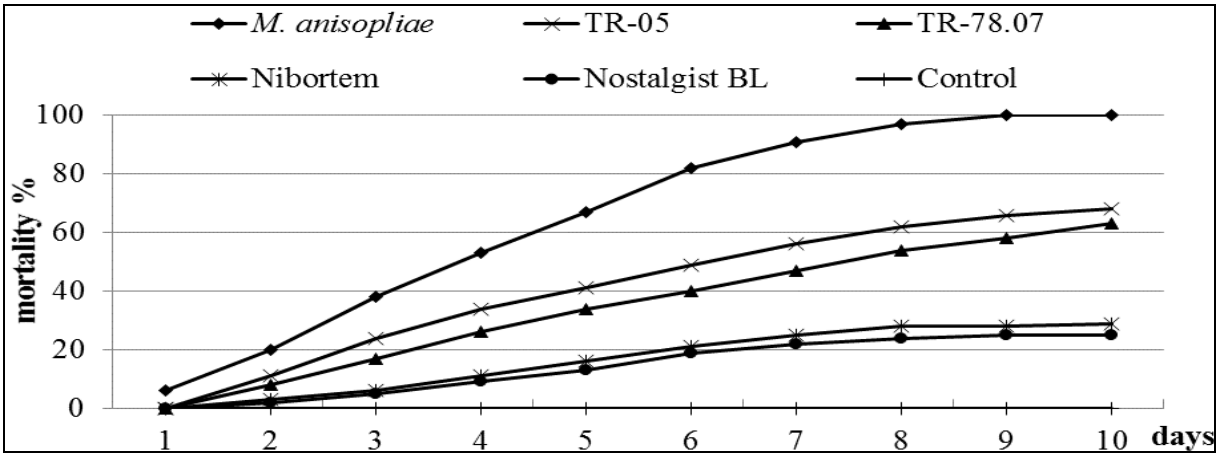

Figure 3: Cumulative mortality percentages of nymphs of *M. coryli* at 25 °C

Isolate of *M. anisopliae*, isolated from *Xylosandrus germanus* (Coleoptera: Curculionidae: Scolytinae), was the most efficacious in controlling nymphs of *M. coryli* at spraying method, especially at 25 °C with 100% mortality after 10 days of treatment. *L. muscarium* (TR-05) was the second most effective isolate in this study that was isolated from infected adults of *P. prasina* (Hemiptera: Pentatomidae). *I. fumosorosea* (TR-78.07) was isolated from infected pupae of *H. cunea*, and its mortality were lesser than *M. anisopliae*. 2 commercially biocontrol products weren't so effective on nymphs of *M. coryli* at spraying method.

Temperature can affect the germination and growth as well as the viability of an entomopathogenic fungus in the laboratory as well as in the field. *Metarhizium anisopliae* is a mesophilic fungus with a temperature range generally between 15 and 35 °C, and the optimum for germination and growth between 25 and 30 °C [31, 47, 36, 5, 18, 48, 13, 30].

Our results showed that first instar nymphs of *M. coryli* were more susceptible to the entomopathogenic fungi at 25 °C than 18 °C. The infection of *M. anisopliae* in *M. coryli* increased as temperature increased. Temperatures have significant effects on germination, radial growth and virulence of the

various isolates. All the fungal isolates grew at 18, 22 and 25 °C the temperatures in our study, and the most suitable temperature 25 °C for all isolates. Ekesi *et al*. (1999) [13] and Dimbi *et al*. (2004) [12] reported that the optimum temperature for radial growth of most isolates of *M. anisopliae* was 25 and 30 °C. Ouedraogo *et al*. (1997) [32] reported that the optimum temperature for vegetative growth of *M. anisopliae* isolates ranged between 25 and 32 °C, with 25 °C being the optimum for most isolates. Kuboka (2013) [23] pointed that the highest sporulation of 10^8 conidia/ml occurred at 25ºC, while the lowest sporulation 10^8 conidia/ml occurred at 15ºC, in addition at 25 and 30ºC, the all isolates induced 100% mortality to adult *F. occidentalis* in six days. Bugeme (2008) [7] pointed that the best fungal germination was observed at 25 and 30 °C, while for the fungal radial growth it was 30 °C on virulent to *Tetranychus evansi*.

4. Conclusion

Use of chemical insecticides is by far the most important tactic applied to control hazelnut pests [5, 41, 45]. Because chemicals are easily available in the open market and are aggressively promoted by commercial manufacturers, they have become the most dominant feature of the hazelnut pest control landscape [3, 16, 26, 33, 43]. But, in recent years there is an increase in organic hazelnut growing in Turkey day by day [44]. In addition, the filbert aphid is highly resistant to chemical pesticides as carbaryl [2], a number of organophosphates [20], and the synthetic pyrethroid, esfenvalerate [19].

The fungal biocontrol agents' entomopathogenic fungi have been widely applied for insect, such as aphids, control. It appears that the use of *M. anisopliae* can provide protection on hazelnuts against aphids and it can be effective as biocontrol agent on *M. coryli.* Spores of *M. anisopliae* can be developing and they can play a significant role in sustainable development of organic agriculture practices.

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