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Pathogenicity of *Metarhizium anisopliae* on larvae of fall webworm, *Hyphantria cunea* (Drury) (Lepidoptera: Arctiidae) at different temperatures

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Abstract
The fall webworm, *Hyphantria cunea* (Lepidoptera: Arctiidae) is a dangerous and destructive pest for forest, fruit trees and ornamental plants. Insecticides are successful to reduce population of this pest, but they cause environmental pollution. In this study, the virulence efficacy of *M. anisopliae* was determined on the fourth instar larvae of *H. cunea* under laboratory conditions. 1×10^6 and 1×10^8 conidial suspensions of *M. anisopliae* grew at 20, 25 and 30°C temperatures. 1×10^8 conidial suspensions of *M. anisopliae* were the most efficacious in controlling larvae of *H. cunea* at spraying method, especially at 30°C with 100 % mortality after 9 days of treatment. 1×10^8 conidial suspensions of *M. anisopliae* more efficacious in controlling larvae of *H. cunea* at 20 and 25°C (63% and 84%) than 1×10^6 conidial suspensions of *M. anisopliae* at 20, 25 and 30°C (34%, 46% and 72%) after 9 days of treatment. This study showed that isolate of *M. anisopliae* has virulent and highly potential for biological control on larvae of *H. cunea.*

Keywords: *Hyphantria cunea, Metarhizium anisopliae*, Different Temperatures, Biological Control

1. Introduction
The fall webworm, *Hyphantria cunea* (Drury) (Lepidoptera: Arctiidae) feeds on hundreds of species of trees, shrubs, ornamentals and annual plants [13]. In Europe, it feeds on 219 species with 103 hosts in Hungary, 85 hosts in Yugoslavia, as well as 48 species in the former Soviet Union [29]. In Japan more than 300 species of plants are hosts including trees, shrubs, weeds, and vegetables [12], in Korea 65 hosts are recorded [31]. The total number of observed hosts is 636 species [29]. This pest has been introduced into west part of Turkey in 1975 and became pest for many fruits and ornamental trees in Turkey [24]. It has a high reproductive rate and ability to spread easily that makes it fairly difficult to be controlled [26].

Today, in controlling of fall webworm especially chemical pesticides are being used. Because the larvae of fall webworm are more sensitive to chemical pesticides, and most traditional chemical pesticides are effective to the pest [11]. Research carried out in Samsun province showed that hazelnut farmers apply insecticides 1-3 times per year [25]. Carbamates and organophosphates are the most-used groups of insecticides. Recently, some synthetic pyrethroids were introduced [26].

Chemical control has faster and significant effect, and can receive immediate control effect under the situations of large disaster area, large occurrence and serious damage [32]. 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 [14, 8, 19]. In addition, chemical control should not be used in large scale for long term, and must be gradually replaced by biological pesticides [32].

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 [23]. The entomopathogenic fungus *Metarhizium anisopliae* can infect 200 species from more than 50 insect families [21], 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 [15]. The aim of this study was to investigate the pathogenicity of the entomopathogenic fungus *M. anisopliae* on larvae of the fall webworm, *Hyphantria cunea*, at different temperatures under laboratory conditions.

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 [4] and identified as *M. anisopliae.*

2.2 Conidial germination assessment
The viability of conidia of *M. anisopliae* was evaluated using a method modified from [10]. A conidial suspension was adjusted to 1×10^4 conidia/mL, and 0.2 mL was sprayed onto 9 cm diameter. 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 [22].

2.3 Inoculum of *M. anisopliae*

Isolate of *M. anisopliae* was 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 1×10^6 and 1×10^8 conidia/mL for each isolate [22].

2.4 Insect rearing

First instar larvae of *H. cunea* were collected from mulberry (*Morus alba* L.) trees in Samsun province, Turkey, during early August of 2016. They were reared as a group of 10 larvae separately on mulberry leaves to get fourth larvae stage in growth chamber (26 ± 1 °C ; 65 ± 5 % RH; 12:12 h L:D) in plastic containers, 10 × 10 × 20 cm.

2.5 Bioassay

Fourth instar larvae of *H. cunea* were placed on mulberry leaves in plastic containers (10 × 10 × 20 cm) containing sterile water-soaked blotters (10 larvae and 5 fresh leaves per plastic container). 1×10^6 and 1×10^8 conidial suspensions of *M. anisopliae* were applied to the fourth instar larvae of *H. cunea* (4 mL per plastic container) using a Potter spray tower (Burkard, Rickmansworth, Hertz UK). Control units were treated with sterile distilled water (4 mL). Each of plastic containers was loosely capped to prevent escape after applications. Plastic containers were incubated at 20±1 °C, 25±1°C and 30±1°C (65±5 % RH and 12:12 h L:D) for 9 days. All plastic containers were inspected daily. Dead larvae of *H. cunea* were counted and removed into empty plastic containers. Mortality of larvae was recorded from 1-9 days after treatment. Leaves were changed after third day and added fresh leaves of mulberry into each plastic container for feeding larvae of *H. cunea.* The experiment was repeated ten times per treatment.

2.6 Statistical analysis

The mortality percentages of larvae for each application were analyzed using "Kruskal-Wallis H Test" (SPSS 21 for Windows). The "Mann-Whitney U Test" is used to compare differences between independent groups. Mortality was considered significantly different at P =0.05.

3. Results

Dose-response relationship was determined for *M. anisopliae* applied to the fourth instar larvae of *H. cunea* at different temperatures under laboratory conditions. The accumulated mortality recorded during 1-9 days showed that 1×10^6 and 1×10^8 conidial suspensions were found effective against larvae (Figure 1, 2). According to our study, significantly different effects on mortality were observed among different doses at different temperatures (p =0.05).

3.1 Efficacy of 1×10^6 conidial suspension at different temperatures

1×10^6 conidial suspension of *M. anisopliae* grew on larvae of *H. cunea* at 20, 25 and 30°C temperatures. Mortality wasn't observed after 3 days of treatment at any temperatures. 1×10^6 conidial suspension of *M. anisopliae* was the most efficacious in controlling larvae of *H. cunea*, especially at 30°C with 72% mortality after 9 days of treatment. Mortality was observed after 9 days of treatment at 20 and 25°C (34% and 46%) but results weren't so effective (Table 1).

3.2 Efficacy of 1×10^8 conidial suspension at different temperatures

1×10^8 conidial suspension of *M. anisopliae* grew on larvae of *H. cunea* at 20, 25 and 30°C temperatures. 1×10^8 conidial suspension killed 47% of population after 5 days of treatment at 30°C. 1×10^8 conidial suspension of *M. anisopliae* was the most efficacious in controlling larvae of *H. cunea*, especially at 30°C with 100 % mortality after 9 days of treatment (Table 2). Mortality rates at 20 and 25 °C (63% and 84%) weren't so effective as at 30°C, but these results were quite good in comparison with 1×10^6 conidial suspension of *M. anisopliae* at same temperatures (34% and 46%).

Table 1: Mortality percentages of larvae of *H. cunea* at different temperatures by using 1×10^6 conidial suspension

Conidia/mL	Days	Mortality percentage of larvae at different temperatures						
		20°C		25°C		30°C		P*
1×10^6	3.	0 ± 0 0 (0-0)	-	0 ± 0 0 (0-0)	-	0 ± 0 0 (0-0)	-	-
	5.	2 ± 1.33 0 (0-10)	c*B**	6 ± 1.63 10 (0-10)	cB	14 ± 1.63 10 (10-20)	cA	<0.001
	7.	12 ± 2.05 10 (0-20)	bC	20 ± 2.62 20 (10-30)	bB	49 ± 1.83 50 (40-60)	bA	<0.001
	9.	34 ± 2.25 35 (20-40)	aC	46 ± 3.11 45 (30-60)	aB	72 ± 2.52 70 (60-80)	aA	<0.001
P*		<0.001		<0.001		<0.001		

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

Table 2: Mortality percentages of larvae of *H. cunea* at different temperatures by using 1×10^8 conidial suspension

Conidia/mL	DAYS	Mortality percentage of larvae at different temperatures						
		20°C		25°C		30°C		P*
1×10^8	3.	0 ± 0 0 (0-0)	-	4 ± 1.63 0 (0-10)	dB	14 ± 1.63 10 (10-20)	cA	0.002
	5.	12 ± 1.33 10 (10-20)	cC	22 ± 2.11 20 (10-30)	cB	47 ± 2.62 50 (30-60)	bA	<0.001
	7.	38 ± 2.11 40 (30-50)	bC	61 ± 2.32 60 (50-70)	bB	92 ± 2.52 90 (80-100)	aA	<0.001
	9.	63 ± 3.0560 (50-80)	aC	84 ± 2.72 80 (70-100)	aB	100 ± 0 100 (100-100)	aA	<0.001
P*		<0.001		<0.001		<0.001		

Larvae in control units survived till the finish of experiment without any mortality. All living larvae in all applications and control units were fed with mulberry leaves in containers. They made cocoons and then transformed into pupae after 10-14 days of application.

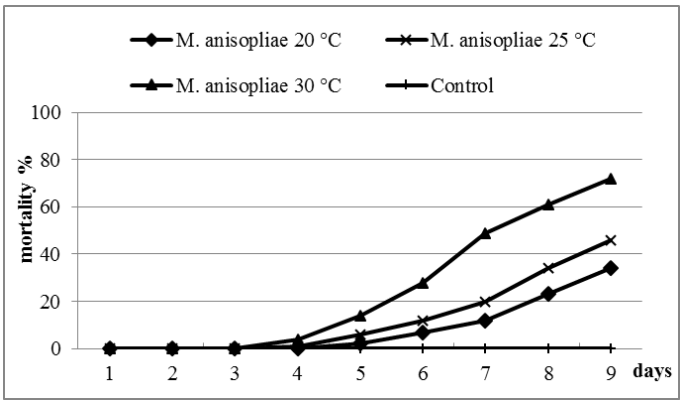

Fig 1: Cumulative mortality percentage of larvae of *H. cunea* at different temperatures by using 1×10^6 conidial suspension

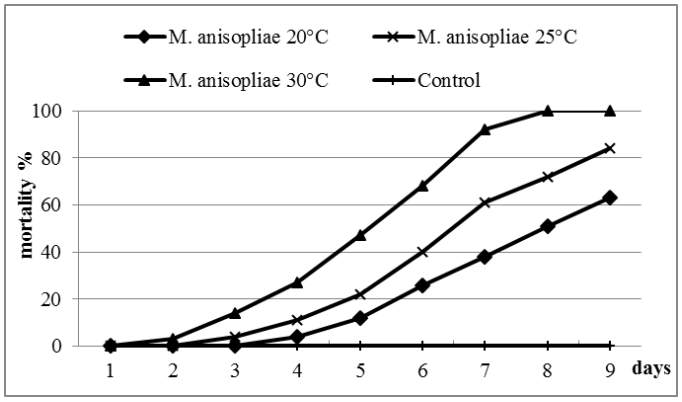

Fig 2: Cumulative mortality percentage of larvae of *H. cunea* at different temperatures by using 1×10^8 conidial suspension

4. Discussion

In our study, the virulence efficacy of *M. anisopliae* was determined on the fourth instar larvae of *H. cunea* under laboratory conditions. 1×10^6 and 1×10^8 conidial suspensions of *M. anisopliae* grew at 20, 25 and 30 °C temperatures. 1×10^8 conidial suspensions of *M. anisopliae* were the most efficacious in controlling larvae of *H. cunea* at spraying method, especially at 30 °C with 100 % mortality after 9 days of treatment. 1×10^8 conidial suspensions of *M. anisopliae* more efficacious in controlling larvae of *H. cunea* at 20 and 25 °C (63% and 84%) than 1×10^6 conidial suspensions of *M. anisopliae* at 20, 25 and 30°C (34%, 46% and 72%) after 9 days of treatment.

Our results showed that fourth instar larvae of *H. cunea* were more susceptible to the *M. anisopliae* at 30 °C than 20 °C by using 1×10^6 and 1×10^8 conidial suspensions. The infection of *M. anisopliae* in *H. cunea* increased as temperature increased, because temperature has significant effects on germination, radial growth and virulence of the various isolates. 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. *M. 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 [17, 28, 20, 1, 7, 30, 6, 16].

Ekesi *et al*. (1999) [6] and Dimbi *et al*. (2004) [5] reported that the optimum temperature for radial growth of most isolates of *M. anisopliae* was 25 and 30 °C. Ouedraogo *et al*. (1997) [18] 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) [9] 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 30 ºC, the all isolates induced 100% mortality to adult *F. occidentalis* in six days. Bugeme (2008) [3] 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*.

5. Conclusion

Entomopathogenic fungi are relatively slow-acting biological control agents compared to synthetic chemical pesticides. Because of this, their success depends in part on the insects' reproductive capacity during the disease incubation period [27]. The entomogenous, hyphomycete fungus *M. anisopliae*, is a pathogenic micro-organism for many insects. Its effectiveness led the researchers to isolate and produce its toxins [2]. From our results, it could be concluded that isolate of *M. anisopliae* contains virulence characters that can be effective as biocontrol agent on larvae of *H. cunea*. Also spores of *M. anisopliae* can be developed as biopesticide and this biopesticides can be used instead of conventional chemical insecticides in controlling larvae of *H. cunea.*

6. References

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