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Pathogenicity of *Beauveria bassiana* 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 *B. bassiana* was determined on the fourth instar larvae of *H. cunea* under laboratory conditions. 1×10^6 and 1×10^8 conidial suspensions of *B. bassiana* grew at 20, 25 and 30°C temperatures. 1×10^8 conidial suspensions of *B. bassiana* were the most efficacious in controlling larvae of *H. cunea* at spraying method, especially at 25°C with 100 % mortality after 9 days of treatment. 1×10^8 conidial suspensions of *B. bassiana* more efficacious in controlling larvae of *H. cunea* at 20 and 30°C (61% and 74%) than 1×10^6 conidial suspensions of *B. bassiana* at 20, 25 and 30°C (23%, 66% and 28%) after 9 days of treatment. This study showed that isolate of *B. bassiana* has virulent and highly potential for biological control on larvae of *H. cunea*.

Keywords: *Hyphantria cunea*, *Beauveria bassiana*, Different Temperatures, Biological Control Agent

1. Introduction
The fall webworm, *Hyphantria cunea* (Drury), has become a serious quarantine pest since its introduction into Eurasia, and its hosts include more than 300 species of plants belonging to 49 families, weeds, etc. [40]. This pest has two generations per year and overwinters as pupae. Adult emergence is between mid-May and mid-June for the first generation and between late July and mid-August for the second generation. The larvae feed on hazelnut leaves and sometimes consume all of the foliage [36, 37].

Most of the hazelnut farmers use pesticides and spray 1-2 times every year for to protect their products from this pest in Turkey [38]. Currently used chemical pesticides are usually relatively cheap and efficient, supply chains exist and growers are equipped to apply those [27]. There is now overwhelming evidence that some of chemicals do pose a potential risk to humans and other life forms and unwanted side effects to the environment [17, 20, 22].

The advantage to using biological products is that they are less likely to negatively impact non target organisms, including people [25]. In recent years, crop protection based on biological control of crop pests with microbial pathogens like virus, bacteria, fungi and nematodes has been recognized as a valuable tool in pest management [3, 32]. Biological control, particularly by entomopathogenic fungi, is important for reducing the population density of pests in Integrated Pest Management (IPM) programs. The entomopathogenic fungi, *Beauveria bassiana* is the most promising biological control agent. Several studies have examined the use of *B. bassiana* as a biological control agent in agriculture [31, 39, 24, 6, 28]. This entomopathogenic fungus has also been intensively studied with the aim of development of commercial mycopesticides for the management of insect pests [10, 11]. *B. bassiana* is considered a broad spectrum biopesticide that can infect a diverse group of insects, and is currently the most ubiquitous mycopesticide used in the U.S. [12]. In this study, we isolated *B. bassiana* and tested its effectiveness on larvae of *H. cunea* at different temperatures. This isolate seems to be a promising biological control agent against this pest.

2. Materials and Methods
2.1 Isolation of *B. bassiana* from insect
The *Beauveria bassiana* was 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±1ºC for fifteen days. The insects with hyphae were then transferred to selective medium for the isolation of *B. bassiana*. The fungus was then grown on Potato dextrose agar (Hi-Media) fortified with 1% yeast extract at 25±1ºC in dark. Single-spore isolates were obtained by serial dilution [13] and identified as *B. bassiana.*

2.2 Conidial germination assessment
The viability of conidia of *B. bassiana* was evaluated using a method modified from [26]. 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 [33].

2.3 Inoculum of *B. bassiana*
Isolate of *B. bassiana* 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 [33].

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 % R.H; 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 *B. bassiana* was 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 *B. bassiana* 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 *B. bassiana* 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 *B. bassiana* was the most efficacious in controlling larvae of *H. cunea*, especially at 25°C with 66 % mortality after 9 days of treatment. Mortality was observed after 9 days of treatment at 20 and 30°C (23 % and 28 %) 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 *B. bassiana* grew on larvae of *H. cunea* at 20, 25 and 30°C temperatures. 1×10^8 conidial suspension killed 60 % of population after 5 days of treatment at 25°C. 1×10^8 conidial suspension of *B. bassiana* was the most efficacious in controlling larvae of *H. cunea*, especially at 25°C with 100 % mortality after 9 days of treatment (Table 2). Mortality rates at 20 and 30°C (61 % and 74 %) weren't so effective, but these results were quite good in comparison with 1×10^6 conidial suspension of *B. bassiana* at same temperatures (23 % and 28 %).

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**	12 ± 1.33 10 (10-20)	cA	2 ± 1.33 0 (0-10)	cB	<0.001
	7.	10 ± 2.11 10 (0-20)	bB	42 ± 2.52 40 (30-50)	bA	12 ± 1.33 10 (10-20)	bB	<0.001
	9.	23 ± 2.11 20 (10-30)	aB	66 ± 3.41 70 (50-80)	aA	28 ± 2.52 30 (20-40)	aB	<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.	1 ± 1 0 (0-10)	dB	16 ± 2.21 15 (10-30)	dA	2 ± 1.33 0 (0-10)	dB	<0.001
	5.	19 ± 1.81 20 (10-30)	cB	60 ± 3.65 60 (40-80)	cA	26 ± 3.06 25 (10-40)	cB	<0.001
	7.	38 ± 2.92 35 (30-50)	bC	94 ± 2.25 95 (80-100)	aA	54 ± 5.11 50 (30-80)	bB	<0.001
	9.	61 ± 3.53 65 (40-70)	aC	100 ± 0 100 (100-100)	aA	74 ± 5.25 70 (50-100)	aB	<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.

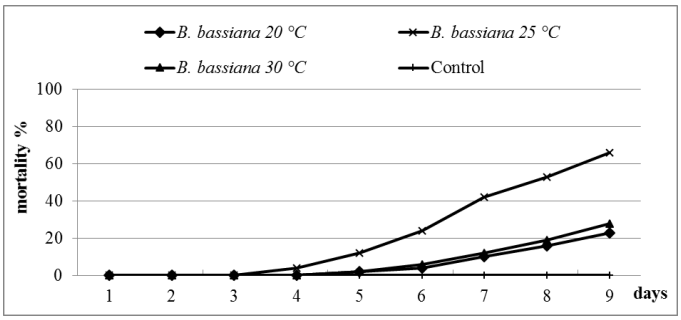

Fig 1: Cumulative mortality percentage of larvae of *H. cunea* at different temperatures by using 1×10^6 conidial suspension

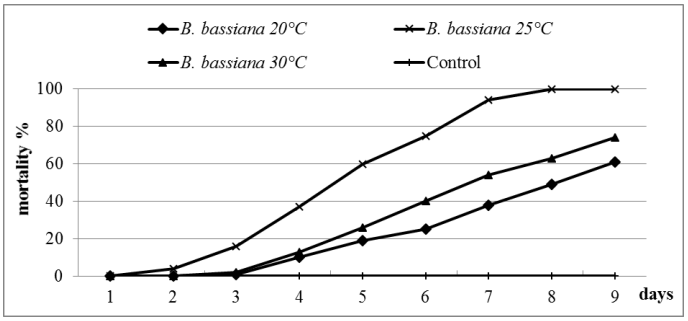

Fig 2: Cumulative mortality percentage of larvae of *H. cunea* at different temperatures by using 1×10^8 conidial suspension

4. Discussion

In our study, the virulence efficacy of *B. bassiana* was determined on the fourth instar larvae of *H. cunea* under laboratory conditions. 1×10^6 and 1×10^8 conidial suspensions of *B. bassiana* grew at 20, 25 and 30°C temperatures. 1×10^8 conidial suspensions of *B. bassiana* were the most efficacious in controlling larvae of *H. cunea* at spraying method, especially at 25°C with 100 % mortality after 9 days of treatment. 1×10^8 conidial suspensions of *B. bassiana* more efficacious in controlling larvae of *H. cunea* at 20 and 30°C (61 % and 74 %) than 1×10^6 conidial suspensions of *B. bassiana* at 20, 25 and 30°C (23 %, 66 % and 28 %) after 9 days of treatment.

Our results showed that fourth instar larvae of *H. cunea* were more susceptible to the *B. bassiana* at 25 °C than 20 and 30 °C by using 1×10^6 and 1×10^8 conidial suspensions. The infection of *B. bassiana* in *H. cunea* decreased as temperature increased. Temperature, humidity and solar radiation are probably the most important environmental factors affecting survival and capability to cause mortality by entomopathogenic fungi [7, 21]. Temperature affects the pathogen-its germination, growth, survival and virulence- the host and the host-pathogen interaction [15, 14, 23].

Nahas and Arai (1987) [30] found that optimal growth rate of *B. bassiana* was obtained at 25°C, in addition the growth rate of *B. bassiana* was close to zero at 37°C. According to Hallsworth and Magan (1999) [19], excellent growth rates could be obtained for *B. bassiana* at 25°C; however, this fungus featured good growth at the 20 to 30°C range. Ekesi *et al*. (1999) [15] and Dimbi *et al*. (2004) [14] reported that the optimum temperature for radial growth of most isolates of *B. bassiana* was 25°C. Similar results have been reported earlier using this entomopathogen against other insects [34, 8]. Athanassiou and Steenburg (2007) [5] reported that humidity and temperature levels for optimal infection for *B. bassiana* is 25°C and 65% RH. Bugeme *et al*. (2008) [9] assessed the effect of temperature on the pathogenicity of *B. bassiana* (isolated from Africa) on spider mite *Tetranychus urticae* Koch and noted that the LT_{50} decreased as the temperature increased, being 9.8 days at 20°C, 4.9 at 25°C and 3.3 at 32°C. The highest mortality (100%) displayed by both the strains of *B. bassiana* was at 26°C, which was in agreement with the experiments of Alexandre *et al*. (2008) [1], where mortality of the *Alphitobius diaperinus* Panzer (Coleoptera: Tenebrionidae). Svedese *et al* (2013) [35] reported that the strains of *B. bassiana* were most pathogenic against *Diatraea saccharali* at 26°C, followed by 32°C than at 20°C, with respective mortalities of 100, 50 and 30.3%. Mwamburi *et al* (2015) [29] reported that the optimal temperature for conidial germination of *B. bassiana* isolates was approximately 25 °C, with an upper limit at 30 °C.

5. Conclusion

Chemical pesticides 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 [2, 18]. However, overreliance and indiscriminate unscientific use of pesticides for longer periods resulted in a series of problems, mainly risk of environmental contamination, loss of biodiversity [4]. The entomopathogenic fungus *B. bassiana* is the most common parasite of insects that has been isolated from soil, litter, and dead or moribund insects in nature [16]. *B. bassiana* can be developing as biopesticite and it can be used instead of conventional chemical insecticides in controlling of larvae of *H. cunea.*

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