Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

J E Z S Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2016; 4(5): 171-176

Received: 25-07-2016
Accepted: 26-08-2016

Onur Aker
University of Ondokuz Mayis, Faculty of Agriculture, Department of Plant Protection, 55139, Samsun, Turkey

Celal Tuncer
University of Ondokuz Mayis, Faculty of Agriculture, Department of Plant Protection, 55139, Samsun, Turkey

Correspondence
Onur Aker
University of Ondokuz Mayis, Faculty of Agriculture, Department of Plant Protection, 55139, Samsun, Turkey

Efficacy of *Metarhizium anisopliae* and Some Entomopathogenic Fungi on Larvae of Fall Webworm, *Hyphantria cunea* (Drury) (Lepidoptera: Arctiidae)

Onur Aker and Celal Tuncer

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. This study determined that *M. anisopliae* was the most efficacious in controlling second and third instars larvae of *H. cunea* at two methods under laboratory conditions. *M. anisopliae* isolate caused to pathogenicity on second instar larvae with the highest lethal effect 85% and the mortality was significantly higher than that caused by any of the other isolates. *M. anisopliae* caused 68.33% mortality and it was the most efficacious in controlling third instar larvae of *H. cunea*. The other isolates (TR-05, TR-78.07 and TR-11) found less effective on larvae of *H. cunea*. 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*, Entomopathogenic Fungi, Biological Control

Introduction
The fall webworm, *Hyphantria cunea* (Drury) (Lepidoptera: Arctiidae), is an insect native to North America which has been accidentally introduced to some areas of Europe and Asia [28] and New Zealand [12]. Fall webworm is the most polyphagous species of the world and about 600 plant species have now been identified as potential hosts [21]. The fall webworm 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. [29]

It has been introduced into west part of Turkey in 1975 and became pest for many fruits and ornamental trees in Turkey [25]. This pest has two generations in a year and especially serious pest of hazelnut plantations along the Black Sea coast line in Turkey and Georgia [27]. It has a high reproductive rate and ability to spread easily that makes it fairly difficult to be controlled [26]. Today, for the control 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 [14]. 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. However, this method should not be used in large scale for long term, and must be gradually replaced by biological pesticides [30].

Entomopathogens are the natural enemies of pests in agroecosystems. Application of entomopathogenic fungi is considered as a priority, as its use will facilitate a decrease in the harmful side effects of chemical pesticides [1]. The entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin has been effective in controlling more than 200 species of insect pests [19]. The application of *M. anisopliae* has several advantages over the conventional chemical pesticides, such as limited harm to humans, honey bees, livestock, and crops [17]. In developing effective replacements for toxic chemicals, entomopathogenic fungi have been considered as an alternative [22]. Adoption of a biological control agent, such as *M. anisopliae*, in integrated pest management (IPM) often results in overall reduction in the total amount of pesticide applied [18].

The aim of this study was to investigate the virulence efficacy of the entomopathogenic fungus *M. anisopliae* and some entomopathogenic fungi on larvae of the fall webworm, *Hyphantria cunea* under conditions of bioassay in laboratory.

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±1ºC 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±1ºC in dark. Single-spore isolates were obtained by serial dilution [8] and identified as *M. anisopliae*.

2.2 Isolation of the 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 [8] and identified as *Lecanicillium muscarium* (isolate TR-05 from *P. prasina*), *Simplicillium lamellicola* (isolate TR-11 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, 11737 and 12177 respectively).

2.3 Conidial germination assessment: The viability of conidia of *M. anisopliae* and the 3 other isolates (TR-05, TR-11 and TR-78.07) were evaluated using a method modified from [13]. 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.

2.4 Inoculum of entomopathogen isolates: Isolate of *M. anisopliae* and the 3 other isolates TR-05, TR-11 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.

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 use in the present study.

2.6 Insect rearing: First instar larvae of *H. cunea* were collected from mulberry (*Morus alba* L.) trees in Samsun province, Turkey, during early August of 2015. They were reared as a group of 10 larvae separately on mulberry leaves to get second and third instar larvae stage in growth chamber (26 ± 2 °C ; 65 ± 10% R.H; 16:8 h L:D) in plastic containers, 10 × 10 × 20 cm.

2.7 Spraying method (a): Second 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). Conidial suspensions of *M. anisopliae* and the 3 other entomopathogenic fungi (TR 05, TR 11, TR 78.07) and the 2 other products (Nibortem and Nostalgist BL) were applied to the second instar larvae of *H. cunea* (2 mL per plastic container) using a Potter spray tower (Burkard, Rickmansworth, Hertz UK). Control units were treated with sterile distilled water (2 mL). Each of plastic containers was loosely capped to prevent escape after applications. Plastic containers were incubated at 26±2°C at 65±10% and a 16:8h L:D photoperiod for 16 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 1-16 days after treatment. Leaves were changed after fourth day and added fresh leaves of mulberry into each plastic container for feeding larvae of *H. cunea.* The experiment was repeated six times per treatment. The same method was designed for third instar larvae and called as spraying method-b. The mortality of larvae was recorded from 1-12 days after treatment in this method.

2.8 Dipping method (a): Mulberry leaves were placed in plastic containers (10 × 10 × 20 cm) containing sterile water-soaked blotters (5 fresh leaves per plastic container). Mulberry leaves were exposed to conidial suspensions of *M. anisopliae* and the 3 other entomopathogenic fungi (TR 05, TR 11, TR 78.07) and the 2 other products (Nibortem and Nostalgist BL) in glass jars for 30 seconds to per plastic containers. Then second instar larvae of *H. cunea* were carefully transferred into each plastic container (10 larvae per plastic container). Control leaves were treated with sterile distilled water. Each of plastic containers were loosely capped to prevent escape after applications. Plastic containers were incubated at 26±2°C at 65±10% and a 16:8h L:D photoperiod for 16 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 1-16 days after treatment. Leaves were changed after fourth day and added fresh leaves of mulberry into per plastic container for to feeding larvae of *H. cunea.* The experiment was repeated six times per treatment. The same method was designed for third instar larvae and called as dipping method-b. The mortality of larvae was recorded from 1-12 days after treatment in this method.

2.9 Statistical analysis: The mortality percentage of larvae 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 $P < 0.001$. Statistical significance was drawn by comparing the p value from "t" test table.

3. Results

Dose-response relationship was determined for *M. anisopliae* and the other entomopathogenic fungi applied to the second and third instar larvae of *H. cunea* at two methods in laboratory conditions. The accumulated mortality recorded during 1-16 days showed that all isolates were found effective against larvae at different rates. According to our study, significantly different effects on mortality were observed among different isolates and commercial products ($p < 0.001$).

M. anisopliae was the most efficacious in controlling second instar larvae of *H. cunea* by two methods after 12 days (at spraying method-a: 66.67% and at dipping method-a: 78.33%) and after 16 days (at spraying method-a: 81.67% and at dipping method-a: 85.00%). The other isolates (TR-05, TR-78.07 and TR-11) found less effective on larvae of *H. cunea* after 12 days (at spraying method-a: 53.33%, 48.33%, 20.00% and at dipping method-a: 36.67%, 25.00%, 11.67%) (Table 1) and after 16 days (at spraying method-a: 63.33%, 58.33%, 25.00% and at dipping method-a: 40.00%, 26.67%, 11.67%) (Table 2). The commercial biocontrol products (Nibortem and Nostalgist BL) were less pathogenic on second instar larvae of *H. cunea* and mean mortality ranged from 11.67% to 25.00% after 16 days.

Table 1: Mortality percentages of second instar larvae of *H. cunea* by using some isolates and commercial products by spraying method-a and dipping method-a.

Isolates and commercial products	Mortality percentage of 2 nd instar larvae after 12 days of application					
	Conidia/mL	Spraying method		Dipping method		Significance
M. anisopliae	1×10^8	66.67 ± 3.33	a	78.33 ± 3.07	a	0.028
TR-05	1×10^8	53.33 ± 5.58	b	36.67 ± 4.94	b	0.049
TR-78.07	1×10^8	48.33 ± 4.77	b	25.00 ± 2.24	c	0.001
TR-11	1×10^8	20.00 ± 3.65	c	11.67 ± 1.67	d	0.065
Nibortem	1×10^8	20.00 ± 3.65	c	16.67 ± 2.11	d	0.448
Nostalgist BL	1×10^8	11.67 ± 1.67	c	11.67 ± 1.67	d	0.999
P*		<0.001		<0.001		

Table 2: Mortality percentages of second instar larvae of *H. cunea* by using some isolates and commercial products by spraying method-a and dipping method-a.

Isolates and commercial products	Mortality percentage of 2 nd instar larvae after 16 days of application					
	Conidia/mL	Spraying method		Dipping method		Significance
M. anisopliae	1×10^8	81.67 ± 3.07	a	85.00 ± 3.42	a	0.485
TR-05	1×10^8	63.33 ± 3.33	b	40.00 ± 3.65	b	0.001
TR-78.07	1×10^8	58.33 ± 3.07	b	26.67 ± 2.11	c	0.001
TR-11	1×10^8	25.00 ± 3.42	c	11.67 ± 1.67	d	0.006
Nibortem	1×10^8	25.00 ± 2.24	c	16.67 ± 2.11	d	0.022
Nostalgist BL	1×10^8	18.33 ± 1.67	c	11.67 ± 1.67	d	0.018
P*		<0.001		<0.001		

M. anisopliae was the most efficacious in controlling third instar larvae of *H. cunea* by two methods (at spraying method-b: 53.33% and at dipping method-b: 68.33%) after 12 days. The other isolates (TR-05, TR-78.07 and TR-11) found less effective on larvae of *H. cunea* (at spraying method-b: 46.67%, 40.00%, 13.33% and at dipping method-b: 23.33%, 18.33%, 3.33%) after 12 days. The commercial biocontrol products (Nibortem and Nostalgist BL) were less pathogenic on third instar larvae of *H. cunea* and mean mortality ranged from 6.67% to 13.33% (Table 3).

Table 3: Mortality percentages of third instar larvae of *H. cunea* by using some isolates and commercial products by spraying method-b and dipping method-b.

Isolates and commercial products	Mortality percentage of 3 nd instar larvae after 12 days of application					
	Conidia/mL	Spraying method		Dipping method		Significance
M. anisopliae	1×10^8	53.33 ± 3.33	a	68.33 ± 7.03	a	0.083
TR-05	1×10^8	46.67 ± 4.94	a	23.33 ± 2.11	b	0.001
TR-78.07	1×10^8	40 ± 7.3	a	18.33 ± 4.77	bc	0.032
TR-11	1×10^8	13.33 ± 3.33	b	3.33 ± 2.11	d	0.03
Nibortem	1×10^8	10 ± 5.16	b	13.33 ± 2.11	bcd	0.563
Nostalgist BL	1×10^8	6.67 ± 3.33	b	6.67 ± 3.33	cd	0.999
P*		<0.001		<0.001		

Larvae in control units survived till the finish of experiment without any mortality at two methods. 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 12-16 days of application. All pupae of *H. cunea* transformed to adult moths in 7-8 days without any mortality.

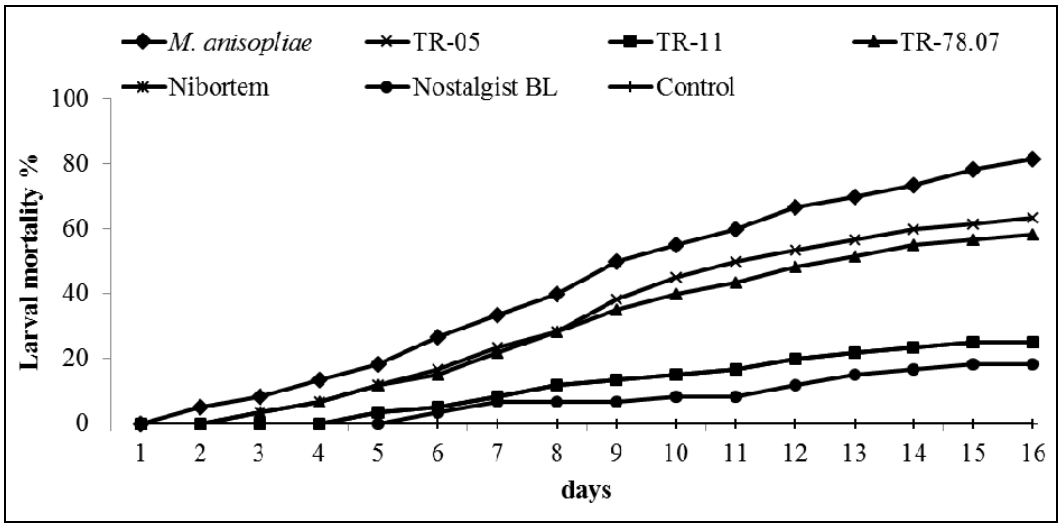

Fig 1: Cumulative mortality percentage of larvae of *H. cunea* at spraying method-a.

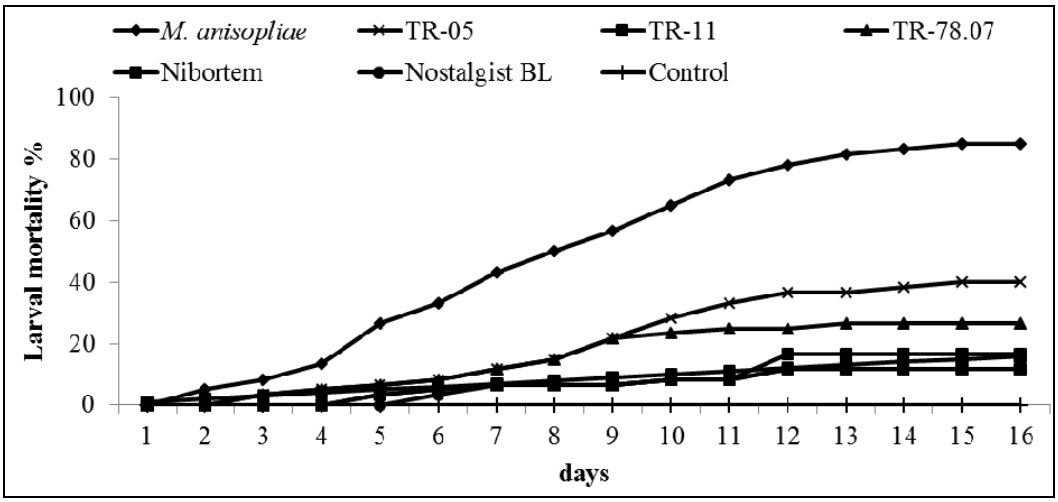

Fig 2: Cumulative mortality percentage of larvae of *H. cunea* at dipping method-a.

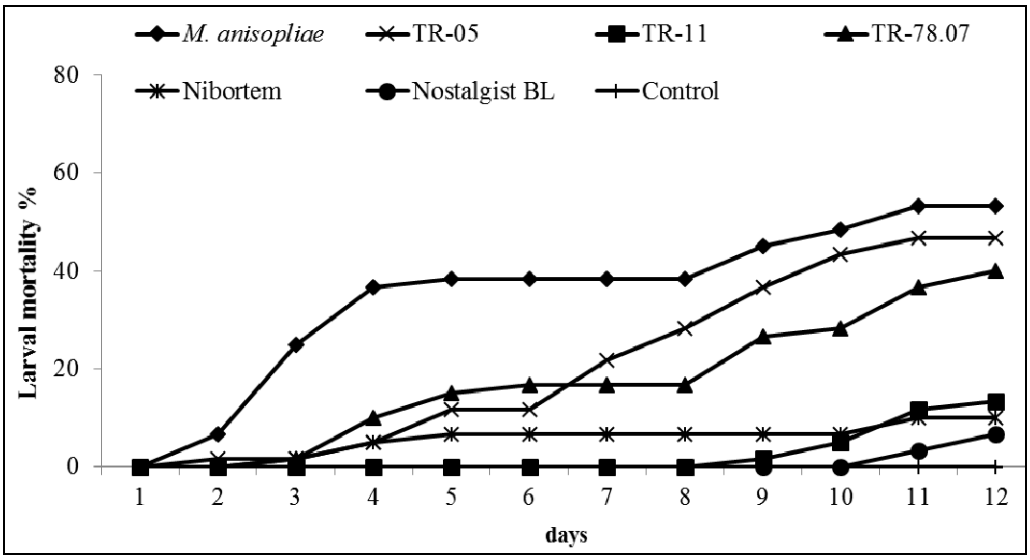

Fig 3: Cumulative mortality percentage of larvae of *H. cunea* at spraying method-b.

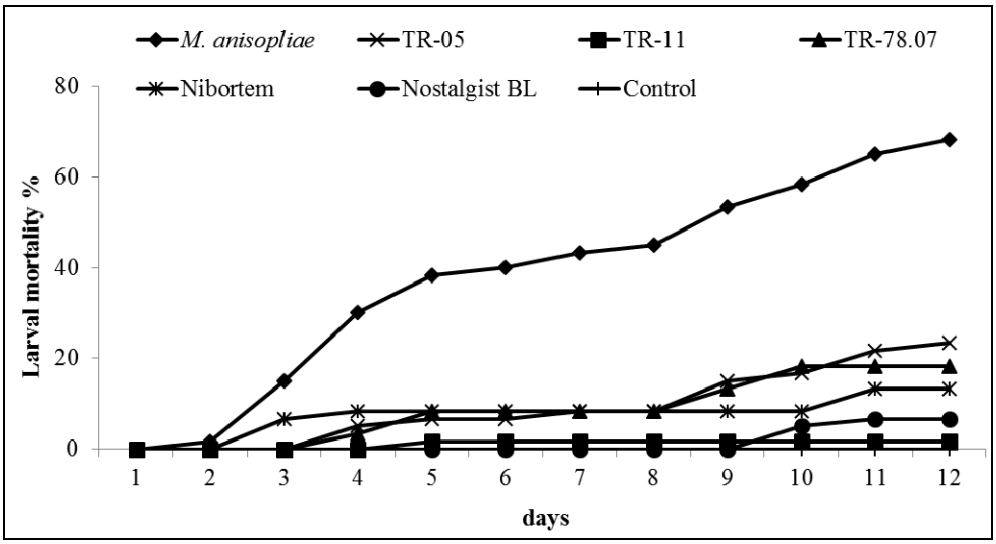

Fig 4: Cumulative mortality percentage of larvae of *H. cunea* at dipping method-b.

4. Discussion

Chemical control is one of the effective and quicker methods in reducing pest population, where farmer obtains spectacular results within a short period. 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, development of insecticide-resistant pest populations, resurgence, outbreaks of the secondary pests, increase in inputs on chemicals and toxicological hazards due to accumulation of pesticide residues in the food chain, etc., ultimately contributing not only to inefficient insect control but also environmental and health hazards [3,11].

So that interest of using the selective biopesticides against phytophagous insects has increased in recent years, particularly in cropping systems that rely on natural enemies as a major component of IPM [20]. Selection of entomopathogenic microorganisms is an important link in the use of effective bio-preparations for protection of plants from insect pests of agricultural crops [24]. 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 [4]. *M. anisopliae* has been studied extensively for the control of wide range pests [6]. There have been many attempts to use this fungus as a practical biocontrol agent against many insect pests, from termites in USA [7] to grasshoppers and locusts in Africa [16].

In our study, the virulence efficacy of *M. anisopliae* and some entomopathogenic fungi were determined on the second and third instar larvae of *H. cunea* under laboratory conditions. Isolate of *M. anisopliae*, isolated from *Xylosandrus germanus* (Coleoptera: Curculionidae: Scolytinae), was the most efficacious in controlling larvae of *H. cunea* at spraying and at dipping method. Moreover, *I. fumosorosea* (TR-78.07) was isolated from infected pupae of *H. cunea*, but its rates of mortality were lesser than *M. anisopliae* on larvae of *H. cunea* at two methods. *L. muscarium* and *S. lamellicola* were isolated from infected adults of *P. prasina* (Hemiptera: Pentatomidae) but *L. muscarium* (TR-05) was the second most effective isolate in this study *S. lamellicola* (TR-11) and 2 commercially biocontrol products weren't so effective on larvae of *H. cunea* at two methods. Results showed that dipping method was more effective on larvae of *H. cunea* than spraying method by using *M. anisopliae* and some entomopathogenic fungi. In addition our results showed that second instar larvae of *H. cunea* were more susceptible to the entomopathogenic fungi than third instar larvae of *H. cunea.*

Burjanadze *et al* [5] reported similar results for *H. cunea* by using *M. anisopliae* (M 7/2) and they managed to kill 68% of 3rd instar larvae population at same suspension (1×10^8). Sapna Bai *et al* [23] reported the 18 isolates were pathogenic to larvae of *Spilarctia obliqua* (Lepidoptera: Arctiidae) by using *M. anisopliae* at 1×10^7 conidia/ml and the mean mortality ranged from 36.0% to 60.2%. *M. anisopliae* isolated from *Schistocerca gregaria* (Orthoptera: Acrididae) caused 100% mortality at 10^8 conidia/ml against second instar larvae of *Spodoptera litura* (Lepidoptera: Noctuidae) [2]. The 2 isolates of *M. anisopliae* at 1×10^7 conidia/ml were pathogenic to *Plutella xylostella* (Lepidoptera: Plutellidae) at different methods the mean mortality ranged from 19.0% to 87.0% [15]. Draganova *et al* [9] showed pathogenicity effects of *M. anisopliae* at 1×10^8 conidia/ml for *Lymantria dispar* (Lepidoptera: Lymantriidae) at different larval stages and the mean mortality ranged from 15.0% to 56.0%. In other study, mortality of larvae of *Spodoptera exigua* (Lepidoptera: Noctuidae) were determined by using *M. anisopliae* at 1×10^8 conidia/ml at different methods and the mean mortality ranged from 3.12% to 87.5% [10].

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 insecticides in controlling of larvae of *H. cunea.*

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