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Effect of *Beauveria bassiana* on *Galleria mellonella* L. (Lepidoptera: Pyralidae) and Its Parasitoid *Trichogramma cacoeciae*

Beauveria bassiana' nın Galleria mellonella L. (Lepidoptera: Pyralidae) ve Parasitoiti Trichogramma cacoeciae Üzerine Etkileri

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Abstract

Beauveria bassiana, which is used for this experiment in order to determine the effects on *Galleria mellonella* and its parasitoid *Trichogramma cacoeciae,* is a naturally occurring pathogens and have been used to control insect pest. The conidia of the fungi germinate on the surface of the insect host, penetrate through the cuticle, spread in the hemolymph, and then resulting in the death of the host. 10^5, 10^6, 10^7 and 10^8 conidia/mL were applied to the last instar of *G. mellonella* by injecting the suspensions. Mortality of *G. mellonella* larvae, effects of *B. bassiana* on emergency of *G. mellonella* eggs and parasitization were investigated. To determine the effect of *B. bassiana* on *T. cacoeciae,* 10^8 conidia/mL were applied to the *G. mellonella* eggs. No mortality was observed in 10^5 and 10^6 conidia/mL of *B. bassiana* injected group, whereas death larvae was determined in 10^7 and 10^8 conidia/mL injected groups. Prevalence of black spots within the integument increased day by day. Hatchability of *G. mellonella* eggs were significantly decreased when *B. bassiana* applied to the *G. mellonella* eggs. No statistical difference was observed in the parasitizing and hatching rates of Galleria mellonella eggs by Trichogramma cacoeciae adult females.

The biosafety of *B. bassiana* against *T. cacoeciae* was determined by monitoring the parasitization and emergence ratio. It was detected *B. bassiana* was safe to this egg parasitoid. However, there is a significant effect on mortality of *G. mellonella* larvae and hatchability of *G. mellonella* eggs.

Keywords: *Beauveria bassiana*, Emergence ratio, *Galleria mellonella*, Survival, *Trichogramma cacoeciae*

Öz

Çalışmada *Galleria mellonella* ve parasitod *Trichogramma cacoeciae üzerine olan etkilerini belirlemek* amacıyla kullanılan *Beauveria bassiana*, doğal olarak oluşan ve zararlı böceklerin kontrolünde kullanılan bir patojendir. Mantarın konidiyumları konak böceğin yüzeyinde gelişmeye başlar, kütikülden geçer, hemolenf içine yayılır ve ardından konağın ölümüyle sonuçlanır. *Galleria mellonella* ve parazitoid *Trichogramma cacoeciae* üzerindeki etkilerinin belirlenmesi amacıyla kullanılmıştır. 10^5, 10^6, 10^7 ve 10^8 spor/mL *B. bassiana, G. mellonella* larvalarına enjekte edilerek uygulanmıştır. *B. bassiana*' nın *G. mellonella* larvalarında mortalite, yumurtalarının açılması ve parazitlenme üzerine olan etkileri araştırılmıştır. *B. bassiana*' nın *T. cacoeciae* üzerindeki etkisini belirlemek için *G. mellonella* yumurtalarına 10^8 spor/mL *B. bassiana* uygulanmıştır. 10^5 ve 10^6 spor/mL *B. bassiana* enjekte edilen grupta mortalite gözlenmezken, 10^7 ve 10^8 spor/mL *B. bassiana* uygulanan gruplarda mortalite meydana geldiği tespit edilmiştir. Integumentteki siyah noktalarda deney süresi boyunca artış meydana geldiği gözlenmiştir. *G. mellonella* yumurtalarının açılma oranı *B. bassiana* uygulaması yapılan gruplarda önemli ölçüde azalmıştır. *T. cacoeciae* ergin dişilerin *G. mellonella* yumurtalarını parazitleme ve yumurtaların açılma oranlarında istatistiki fark gözlenmemiştir.

Sonuç olarak, *B. bassiana*' nın yumurta parazitoidine karşı güvenli olduğu, buna karşın *G. mellonella* larvalarının mortalitesi ve yumurtaların açılma oranı üzerinde önemli bir etkisi olduğu belirlenmiştir.

Anahtar Kelimeler: *Beauveria bassiana*, Yumurtadan çıkma oranı, *Galleria mellonella*, Sağkalım, *Trichogramma cacoeciae*

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1. Introduction

Entomopathogenic fungi are the most common pathogens that cause disease in agriculture and forest pests and known to be one of the most important biological control groups due to being cheap and easy to apply and able to enter the host actively when they find suitable conditions for spore germination, (Demirci et al. 2011).

Beauveria bassiana (Balsamo) (Ascomycota: Hypocreales: Clavicipitaceae) is a widely-distributed fungus, which affects a broad variety of insect species and is used as a biological pesticide (Dembilio et al. 2010; Fuguet and Vey 2004; Wojda et al. 2009; Xiong et al. 2013). *Beauveria* species attack their host insects percutaneously. The infection pathway consists of the following steps; attachment of the spore to the insect cuticle, spore germination on cuticle, penetration through the cuticle, overcoming the host immune response, proliferation within the host, saprophytic outgrowth from the dead host and production of new conidia (de Fariaa and Wraight, 2007; Keswani et al. 2013). Due to being the first barrier encountered by fungi, insect cuticle synthesize a wide variety of extracellular enzymes involved in the degradation of protein, chitin, and lipids which are the principal components of the insect's cuticle. After the death of the insect, the fungus produces thousands of new spores on the dead body which disperse and continue their cycle on new hosts (Pedrini et al. 2007; Schrank and Vainstein 2010; Pelizza et al. 2017).

Due to the ease of breeding on alternative hosts, high aggressive parasitism and wide geographic distribution, *Trichogramma* species are widely used agents for biological control (Souza et al. 2016). *Trichogramma cacoeciae* (Hymenoptera: Trichogrammatidae) is a species of parasitoids of insect eggs that has been reported by several researchers to be efficient in comparison to several other parasitoids of insect pests (Potrich et al. 2015).

Investigations regarding the effects of chemical insecticides on parasitoids are common, however there are few studies on the efficiency of entomopathogenic fungi on parasitoids and combined use of biological control methods. The aim of the study is to know about interactions between biological control agents, used in combination or simultaneously and understand the effects of entomopathogenic fungi by using *Galleria mellonella* L. (Lepidoptera: Pyralidae) as host organism.

2. Materials-Methods

2.1. Insect Rearing

G. mellonella larvae were reared in the dark at 30 °C and %65 humidity in an incubator. Larvae were reared during the first and second instars with pollen and wax, afterwards in an artificial diet were prepared with a mixture of glycerol, honey, water, dry dog food, and wheat bran.

Adult *T. cacoeciae* were used in this experiment. Parasitoids were reared on *G. mellonella* eggs in the laboratory conditions at 23±1 °C and 16:8 photoperiods. Adults were fed with a droplet of a honey solution.

2.2. Culture of *B. bassiana*

The entomopathogenic fungus *B. bassiana* was cultivated and maintained on a rich medium, potato dextrose agar (PDA; Biokar), incubated at 25±1 °C, under dark conditions, for 15 days to allow fungal growth and conidial production.

Conidia were harvested in PBS and %1 (v/v) Tween 20, by scrapping the surface of PDA culture with a sterile loop. The different conidia concentrations were estimated by using Neubauer haemocytometer and the different concentration for each culture (10^5, 10^6, 10^7, 10^8 conidia/mL) were prepared and tested for its efficacy on last instar larvae of *G. mellonella* and *T. cacoeciae*.

2.3. Mortality of *G. mellonella* Larvae

Last instar *G. mellonella* used for the experiment were selected to be similar in size (250-270 mg). Four different suspensions (10^5, 10^6, 10^7, 10^8 conidia/mL) of *B. bassiana* were applied to last larvae instar of *G. mellonella* by injecting 10 μl of suspension using a Hamilton syringe, at the last left pro-leg of larvae. Before injection the proleg was disinfected with %70 alcohol by using a swab. After the treatment with the different concentrations, the larvae were put in 15 mm Petri dishes and incubated, under dark conditions at 30±1 °C for 7 days. For the control, a PBS and Tween 20 mixture was injected. Fifteen larvae were used for each experiment and all the trials were done in triplicates. Larvae mortality was checked daily with a paintbrush. Larvae was considered dead when there was no movement in response to touch. Also, a change on the larvae colour was investigated.

2.4. Spray Bioassay

A *B. bassiana* suspension of 10^8 conidia/mL were tested for its effect on *G. mellonella* eggs measured by the emergence ratio of L1 larvae and for its safety on the egg parasitoid of *T. cacoeciae* and measured by the number of parasitized eggs and emergence ratio.

Fresh *G. mellonella* eggs (12 h old) were collected from the top of the rearing pots and divided into little pieces each having at least 40 eggs. For determining the effect of *B. bassiana* on parasitization, the highest concentration of spores (10^8 conidia/mL) were sprayed on the *G. mellonella* eggs, air-dried and then placed in the tubes with one *T. cacoeciae* female. For the control, egg cards were sprayed using the same methodology but using water. After 24h, egg cards were taken from the tubes and put into different tubes to wait hatching or parasitoid emergence. The number of parasitized eggs were evaluated by the number of eggs become dark (Figure 1). The emergence ratio was evaluated by the number of adults emerged in relation to the number of dark eggs. Each modality had 20 replicates. The experiment was conducted at room temperature of 25±2 ºC, 60% RH and photoperiod 16:8.

Figure 1. Parasitized *G. mellonella* eggs.

2.5. Statistical Analysis

The Kaplan-Meier (SPSS 21 Statistical Programme) test was used for the statistical significance of differences in survival of *G. mellonella* larvae between different conidia concentrations. T test (SPSS 21 Statistical programme) was used for the statistical significance of differences in the effects of *B. bassiana* on egg hatching, number of parasited eggs and adult parasitoid emergence ratio in relation to control. For emergence ratio data was transformed by prior to data analysis. Differences indicated with a P value of less than <0.05 were considered significant.

3. Results and Discussion

3.1. Injection Bioassay

The different spore suspensions (10^5, 10^6, 10^7, and 10^8 conidia/mL) of *B. bassiana* which was cultivated in PDA media were applied on wax moth *G. mellonella*. Physical and motile changes were determined in larvae post-infection with fungal cells and the highest mortality was observed at 10^7 and 10^8 conidia/mL suspensions due to *B. bassiana* infection as compared to the control larvae. As shown in Fig. 2., the larvae of the 10^7 and 10^8 conidia/mL of *B. bassiana* injected groups were killed faster compared to the larvae injected with PBS (control), 10^5 and 10^6 conidia/mL. No mortality was observed in 10^5 and 10^6 conidia/mL of *B. bassiana* injected groups. Death of larvae was observed in 10^8 conidia/mL injected group within 72h but this was delayed to 120h with 10^7 conidia/mL of *B. bassiana* injected group (Figure 2).

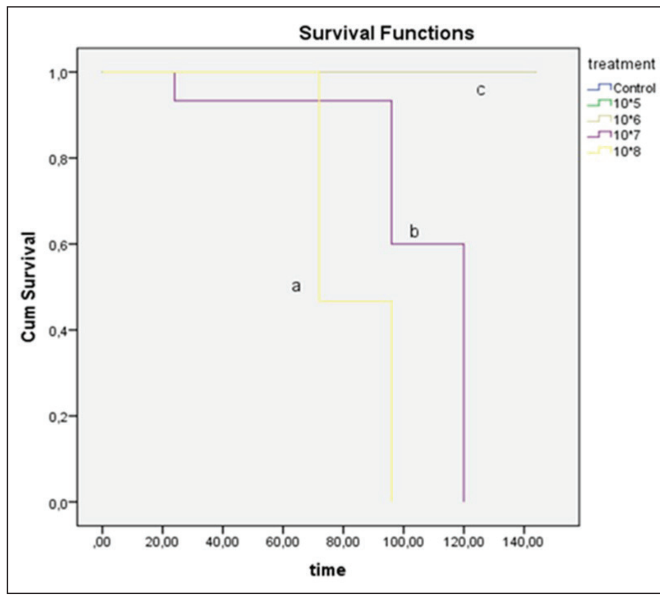

Figure 2. Killing of *G. mellonella* by *B. bassiana*. Kaplan-Meier plots of the control, 10^5, 10^6 ,10^7 and 10^8 conidia/mL of *B. bassiana* survival after injection. Little letters represent statistical differences for each treatments of exposure ($p<0.05$).

Overall, it is obvious that high concentrations of *B. bassiana* reduced survival. Ibrahim et al. (2016) found that *B. bassiana* has 98% larval mortality with lethal time of 1.7 days on *G. mellonella* by using 10^6, 10^7 and 10^8 spores/mL of *B. bassiana*. Nedveckyte et al. (2011) also detected *B. bassiana* lead to 100 % mortality within 12 days on *Bupalus piniaria* (L.) larvae at a concentration of 1x10^8 spores/mL. It was determined that the virulence of *B. bassiana* against second, third and fourth instar larvae of *Spodoptera litura* by using 2.03x10^8, 4.03x10^6 and 1.4x10^5 spores/mL concentrations, and all the treatments resulted in significantly higher mortality than control (Kaur et al. 2011). According to Wraight et al. (2010) Lepidopteran insects such as *Plutella*

Figure 3. Black spots as the immune response indicating direct penetration of *B. bassiana.*

xylostella, *Obstrinia nubilalis*, *Helicoverpa zea* and *Spodoptera frugiperda* are highly susceptible to *B. bassiana* strains. Later, Correa-Cuadros et al. (2014) also indicated that *B. bassiana* had a mortality of 95, %33 ar 1x10^5 conidia/cm^2 on *P. xylostella* larvae. These findings are also supported by another study on *G. mellonell*a that demonstrated that the mortality of greater wax moth was %0, %8 and %10 at 24,72 and 120h respectively following infection with *B. bassiana*. By the fifteen days, total mortality was %52 (Chertkova et al. 2018).

According to the morphological changes, prevalence of black spots within the integument increased day by day. Death of the larvae was followed by a change in the body colour to brunny (Figure 3).

According to Hussein et al. (2012), the cuticle of the treated larvae became dark and black-spotted due to excessive melanization, indicating direct attack of the fungus on the defence system of the insect. Xiong et al. (2013) found that *B. bassiana* infected the host larvae of *Carposina sasakii* (Lepidoptera: Carposinidae) mainly by penetrating the integument, and dark spots appeared on the cuticle due to the appearance of melanisation in the hemocoel. After overcoming the host's defense system, the pathogen grew and reproduced in the hemocoel.

3.2. *Effect of B. bassiana on Emergence of G. mellonella Eggs and Adult of T. cacoeciae, Parasitization*

Hatchability of *G. mellonella* eggs were significantly decreased when *B. bassiana* applied to the *G. mellonella* eggs (Table 1; p<0.05). Kaur et al. (2011) determined that *B. bassiana* induced egg hatchability of *S. litura*. In another study, it was shown that *Metarhizium anisopliae* has a potential effect on both eggs and first larval stage of *Tuta absoluta*, although *B. bassiana* is effective just on egg stages (Inanlı et al. 2012). Yuksel et al. (2017) determined that *B. bassiana* has a high pathogenicity in egg term of *T. absoluta* in vitro conditions. It was also found that egg hatchability ratio was 86 % in control group, while it is 18 % in *B. bassiana* applied group. We were detected the similar results with other studies. It was found that there is a significant difference about emergence of *G. mellonella* eggs between *B. bassiana* applied and the control groups. We assumed that the larvae couldn't be grown inside the eggs or couldn't be emerged due to the fungal infection.

Parasitism and emergence ratio were unaffected by *B. bassiana* (Table 1; p>0.05). Amaro et al. (2015) found that *B. bassiana* had no significant effect on adult emergence, parasitisim and progeny viability. Otherwise, according to Potrich et al. (2009) *Metarhizium anisopliae* decreased *Trichogramma pretiosum* emergence and caused mortality. dos Santos Junior et al. (2006) found that *B. bassiana* could cause a significant reduction in parasitism of larvae of *Plutella xylostella* L. by *Oomyzus sokolowskii* (Kurdjumov). However, Greathead and Prior (1990) maintained that there are no signs of intolerance adverse effects by *Metarhizium flavouiride* on a number of non-target species.

Table 1. Effects of *B. bassiana* on *G. mellonella* and *T. cacoeciae*. Little letters represent statistical differences for each treatments of exposure (p<0.05). X ± sx = mean ± standard error.

	Variable		Control	
Hatching ratio	19.4 ± 4.23	b	37.4 ± 3.42	a
Number of parasitized eggs	15.0 ± 1.91	a	11.4 ± 1.48	a
Parasitoid emergence ratio	0.40 ± 0.03	a	0.65 ± 0.05	a

The use of chemical insecticides induces adverse effects to the beneficial insects and non-target organisms in the ecosystem due to its toxins. *B. bassiana* is a biopesticide that has high virulence against pests, while little or no virulence against non-target organisms such as parasitoids. This is particularly important in the case of pest control, since *B. bassiana* present low risk for humans and other mammals, and it easily cultured and formulated.

In the present study, when we applied *B. bassiana* on *G. mellonella* larvae and eggs, we were determined that there is an important effect on mortality of *G. mellonella* larvae, hatchability of *G. mellonella* eggs, however there is no significant effect on parasitism and emergence ratio of *T. cacoeciae*, so that this fungus could be used in pest management studies in order to control the pests in the fields.

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