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The Combine Effects of Eicosanoid Biosynthesis Inhibitors and Different Isolates of *Metarhizium anisopliae* (Metschn.) and *Beauveria bassiana* (Balsamo) (Deuteromycota: Hyphomycetes) on the Mortality of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) Larvae

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ABSTRACT
The effects of entomopathogen fungus isolates on the mortality of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) larvae were increased and accelerated when co-administered with eicosanoid inhibitors (EBIs) (Dexamethasone, Indomethacin, Esculetin Phenidone Ibuprofen and Naproxen). The mode of action of these compounds are different. When eicosanoid inhibitors were applied to *S. littoralis* larvae together with entomopathogenic fungus isolates (*Beauveria* 6646 and *Metarhizium* 3293), they accelerated deaths of the insect and consequently increased mortality rates. In addition, when different doses of Phenidone, one of the eicosanoid inhibitors, were administered to the larvae with the fungal isolates, there was a significant increase in mortality due to the dose of Phenidone. These results showed that the application of eicosanoid inhibitors with fungal agents to *S. littoralis* larvae increased the effectiveness of these potential microbial control agents.

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Anahtar Kelimeler
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Metarhizium Anisopliae ve Beauveria Bassiana'nın Farklı İzolatlarının Eikosanoid Biyosentezi İnhibitörleri ile Birlikte Spodoptera Littoralis Larvalarına Uygulandığında Larvalar Üzerindeki Ölüm Etkisi

ÖZET
Entomopatojen fungus izolatlarının *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) larvalarının ölümü üzerindeki etkileri eikosanoid inhibitörleri (EBI'ler) (Deksametazon, İndometasin, Esculetin Fenidon Ibuprofen ve Naproksen) ile birlikte larvalara uygulandığında, larvaların ölüm oranı artmış ve hızlanmıştır. Eikosanoid inhibitörlerinin etki mekanizması birbirlerinden farklıdır. Entomopatojen fungus izolatları (*Beauveria* 6646 ve *Metarhizium* 3293) ile eikosanoid inhibitörleri birlikte *S. littoralis* larvalarına uygulandığında, böceğin ölüm oranlarını önemli derecede artırdığı gibi aynı zamanda böcek ölümleri hızlanmıştır. Ek olarak, bahsedilen fungus izolatları ile larvalara farklı dozlarda eikosanoid inhibitörlerinden Phenidone uygulandığında, Phenidone dozuna bağlı olarak larva ölüm oranında önemli bir artış olmuştur. Bu sonuçlar, eikosanoid inhibitörlerinin fungal etmenlerle birlikte *S. littoralis* larvalarına uygulanmasının, potansiyel mikrobiyal kontrol etmenlerinin bu zararlı üzerinde etkinliğini arttırdığını göstermiştir.

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INTRODUCTION

Insects represent two types of immunity to microbial infections which are humoral and hemocytic immunity (Gilles-pie et al., 1997). Humoral immune reactions include induced biosynthesis of anti-microbial proteins (Leulier et al., 2003; Stanley and Miller, 2006). Hemolytic immune reactions include direct interactions between hemocytes and germs (Stanley and Miller, 2006). These immune

functions are well known and provide information on the signaling mechanisms responsible for the limits of contemporary research to mediate and coordinate insect immunity. Stanley-Samuelson et al. (1991) found that eicosanoids take role on insect cells and are responsible for clearing microbial infections from hemolymph circulation of insects. This finding initiated a more detailed study to determine which of the few cell defense reactions is due to eicosanoid biosynthesis. Because nodulation is dominant cellular immunity in insect to bacterial infections, it has been suggested that eicosanoids take part in nodulation reactions to bacterial infections in insect (Miller et al., 1994). So far many researches have been conducted many studies in this area (Stanley, 2006; Stanley and Miller, 2006). All experimental studies supported the mentioned hypothesis. Eicosanoids affect various aspects of insects' immunity. Mandato et al. (1997) found that eicosanoids mediated phagocytosis, which is another cellular immune reaction in insects, in larvae of *Galleria mellonella* (L.) (Lepidoptera: Pyralidae). Dean et al. (2002) and Lord et al. (2002) suggested that eicosanoids mediate the cellular response of *Manduca sexta* (L.) (Lepidoptera: Sphingidae) against the fungal pathogens *Beauveria bassiana* and *Metarhizium anisopliae*, another role of eicosanoids in insect cellular immunity. Connick et al. (2001) tested the role of eicosanoid biosynthesis inhibitors in combination with *Serratia marcescens* (Bizio) for insect pest control. The results showed that the mortality of termites was increased when the bacteria were administered with eicosanoid biosynthesis inhibitors. In addition, Tunaz and Küsek (2012); (2015) showed that when the bacteria, *S. marcescens* was applied together with eicosanoid biosynthesis inhibitors to *Blattella germanica* (L.) (Blattodea: Blattellidae) adults and *Spodoptera littoralis* larvae, the mortality of adults and larvae were increased. Also, Tunaz (2006) tested the effect of different fungal species with EBIs on the nodule formation and the mortality of Pieris brassicae (L.) (Lepidoptera: Pieridae) larvae. Again, when the fungi were applied to *P. brassicae* larvae together with eicosanoid biosynthesis inhibitors, the mortality of *P. brassicae* larvae were increased in an accelerating manner. The eicosanoid hypothesis is also supported by another study on humoral immunity. Morishima et al. (1997) found that the biosynthesis of the anti-bacterial proteins was also related to eicosanoids in the silkworm, *Bombyx mori* (L.) (Lepidoptera: Bombycidae).

Hence, the aim of this study was to determine the effect of different fungal isolates on the mortality of *Spodoptera littoralis* larvae and to determine whether mortality of *S. littoralis* larvae will increase or not, when they were injected with EBIs plus different fungal isolates.

MATERIAL and METHODS

Organisms

Spodoptera littoralis was reared on an artificial diet (2600 ml distil water, 38 g agar, 300 g corn flour, 20 g casein, 120 g wheat embryo, 100 g yeast, 8 g sorbic acid, 14 g Wesson salt, 18 g ascorbic acid, 4 g nipagin, 80 mg vitamin complex and 600 mg streptomisin) and they were kept in the laboratory at 25±2 °C and 65±5% relative humidity (RH). It was tested the larvae (5. instars) for each bioassays at 25 ± 2 °C and 65 ±5 % RH.

Five entomopathogenic fungi, *M. anisopliae* (isolates ARSEF 2775, ARSEF 3293) and *B. bassiana* (isolates ARSEF 1512, ARSEF 3288, ARSEF 6646) were used in this study. The isolates were grown at 25 °C on potato dextrose agar (PDA) in the plates for 30 days. Conidia were harvested from these cultures in sterile distilled water containing 0.1 % Tween 80 and the suspension was vortexed vigorously. After passing through sterile cheese cloth, the suspension was vortexed again and the concentration was determined by spore counting using a hemocytometer under a phase contrast microscope. Required concentration for experiments was achieved by dilution.

Reagents

Eicosanoid biosynthesis inhibitors; dexamethasone, Ibuprofen, indomethacin, naproxen, phenidone and esculetin were provided from Sigma (St. Louis, MO).

Influence of Different Isolates of *M. anisopliae* and *B.bassiana* on the Mortality of *S. littoralis* Larvae

Larvae were applied for ten seconds with individual isolates of *M. anisopliae* (2735 and 3293) and *B. bassiana* (1512, 3888 and 6646) ($1x10^7$ conidia/ml for each isolates). Control insects were applied with 0.02 % Tween 80 solution. After application, the larvae were kept at room temperature. Three replications were used for each test and ten larvae were used for each replicate. Mortality was assessed at 3, 5, 7 and 9 days after injections.

Effects of Eicosanoid Biosynthesis Inhibitors on the Mortality of *S. littoralis* Larvae When Co-Injected With *M. anisoplia* Isolate 3293 and *B. bassiana* Isolate 6646

After dividing *S. littoralis* larvae into groups, we injected 104 µg standard dosage (in 4 µl ethanol EtOH) either inhibitor dexamethasone (phospholipase A2 (PLA2)), naproxen, ibuprofen, indomethacin (cyclooxygenase inhibitors), phenidone (both cyclooxygenase and lipoxygenase inhibitor) or esculetin (lipoxygenase inhibitor) to each individuals in the relevant group. Control insects were injected only with 4 ul of EtOH. Following injections, the larvae were injected with fungal spores at $1x10^7$ conidia/ml concentration in 5 µl 0.021 % Tween 80 solution. The

larvae were maintained at room temperature after injection as described. Each test was replicated three times and ten larvae were used for each replicate. At selected times dead/alive larvae were counted and recorded.

Influence of Phenidone (Eicosanoid Biosynthesis İnhibitor) Dosages on The Mortality of *S. littoralis* Larvae When Co-Injected With *M. anisoplia* and *B. bassiana*

Individuals in 5 larval groups were injected with 4 µl of ethanol, or 52, 104, 156, 208 µg of phenidone in 4 µl ethanol, then inoculated with *M. anisoplia* isolate 3293 and *B. bassiana* isolate 6646 at a standard concentration. Mortality was evaluated after 24 hours.

Statistical Analysis

The data obtained from eicosanoid trials were subjected to Abbott correction formula for deaths in the control group. The proportional data were subjected to analysis of variance (ANOVA) after arcsine correction and the means were separated by Tukey's test at a 5% significance level.

RESULTS

Mortality Effect of The Fungal Isolates on *S. littoralis* Larvae

Mortality effect of the fungal isolates on larvae is shown in Table 1. Compared to controls, all fungus isolates caused higher mortality at each time point. At day 9, control caused no larval mortality of *S. littoralis* whereas the isolate 3293 of *M. anisopliae* caused approximately 63 % mortality of the larvae. Similarly, at day 9, control caused no larval mortality of *S. littoralis* whereas the isolate 6646 of *B. bassiana* caused approximately 67 % mortality of the larvae. The other isolates caused lower mortality of *S. littoralis* larvae.

Combine Effect of Eicosonoid Inhibitors and Entomopathogenic Fungi on The Death of *Spodoptera littoralis* Larvae

According to the results, if the eicosonoid inhibitors were added to the fungus isolates (*Metarhizium* isolate 3293 and *Beauveria* isolate 6646), mortality rates of *S. littoralis* were found to be higher than the fungus isolates alone, and death time of the larvae was shortened. When analyzed by Figure 1 and 2, *Metarhizium* isolate 3293 and *Beauveria* isolate 6646 conidia plus phenidone, an eikosanoid inhibitor, killed approximately 80 % of the larvae after 24 hours when applied to *S. littoralis* larvae. On the other hand, *Metarhizium* isolate 3293 and *Beauveria* isolate 6646 alone killed 10 % of the larvae at the end of 24 hours when applied to the larvae. Again, as shown in figure 1 and 2, when all eicosanoid inhibitors (Dexamethasone, Indomethacin, Esculetin Phenidone Ibuprofen and Naproxen) combined with either *Metarhizium* isolate 3293 or *Beauveria* isolate 6646 were applied to *S. littoralis* larvae, the mortality rate of the insect increased starting from day two comparing with application of fungal isolates alone. When eicosanoid inhibitors were applied to *S. littoralis* larvae with fungus isolates, they showed differences in terms of shortening the duration of death. When the conidia of either isolates were applied to the larvae together with the inhibitor phenidone, the larvae were killed in a shorter time.

Table 1. Mortality rates (%) (± standart error) of *S. littoralis* larvae as a result of the application of different fungal isolates.

Fungal isolates	Times (days)			
	3	5	7	9
B. bassiana 6646	33.33±3.33 a	60.00±0.00 a	66.67±3.33 a	66.67±3.33 a
B. bassiana 1512	10.00±5.77 abcd	33.33±8.82 bc	33.33±8.82 bc	33.33±8.82 bc
M. anisopliae 2735	3.33±3.33 cd	16.67±3.33 c	20.00±5.77 c	20.00±5.77 c
M. anisopliae 3293	23.33±3.33 ab	50.00±0.00 ab	63.33±3.33 a	63.33±3.33 a
B. bassiana 3288	6.67±3.33 bcd	26.67±6.67 bc	33.33±8.82 bc	33.33±8.82 bc
Control	0.00±0.00 d	0.00±0.00 d	0.00±0.00 d	0.00±0.00 d

The values are the mean of three replications, and the different letters in each column indicate a statistical difference between the mortality rates according to Tukey test (P≤ 0,05).

Mortality Effect of Different Doses of Phenidone (Eicosonoid Biosynthesis Inhibitor) With Entomopathogenic Fungi on *Spodoptera littoralis* Larvae

Figure 3 and 4 present the results of the application of a constant concentration of the conidia of *Metarhizium* isolate 3293 and *Beauveria* isolate 6646 together with different dosages of phenidone. The result showed that increasing phenidone (eicosanoid biosynthesis inhibitor) dosages was associated with increasing mortality of the larvae at the end of 24 hours (Figure 3 and 4).

DISCUSSION

This study demonstrated that inhibition of eicosanoids associated with insect pathology and, therefore, microbial challenge, has accelerated biological activity and increased mortality rates, particularly as a result of entomopathogenic fungus and fungus administration. When eicosanoid inhibitors were applied to *S. littoralis* larvae with entomopathogenic fungus isolates (*Beauveria* 6646 and *Metarhizium* 3293) together, they accelerated deaths of the insect and increased mortality rates of the insect. Eicosanoid inhibitors were administered to the larvae with fungus isolates a higher rate of death was achieved compared to the larvae without eicosanoid inhibitors. In addition, there was a significant increase in mortality due to the dosages of phenidone, one of the eicosanoid inhibitors. These results showed that the application of eicosanoid inhibitors with fungal agents to *S. littoralis* larvae increased the effectiveness of these pathogens on insects.

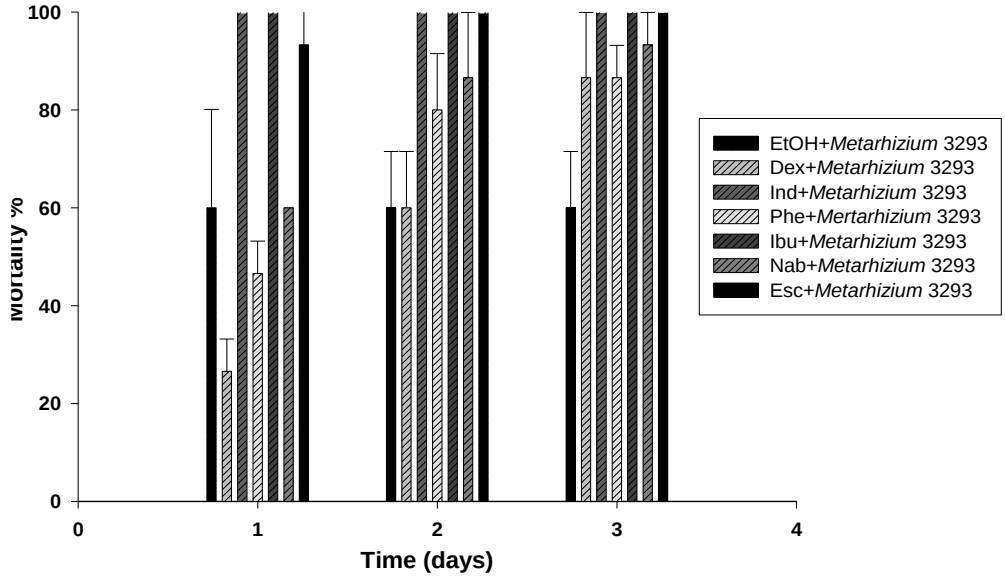

Figure 1. Effect of eicosanoid biosynthesis inhibitors on the mortality of *Spodoptera littoralis* larvae infected with *Metarhizium anisoplia* (isolate 3293). Mortality was assessed at selected times after applications. The points indicate percentage mortality on days post-application.

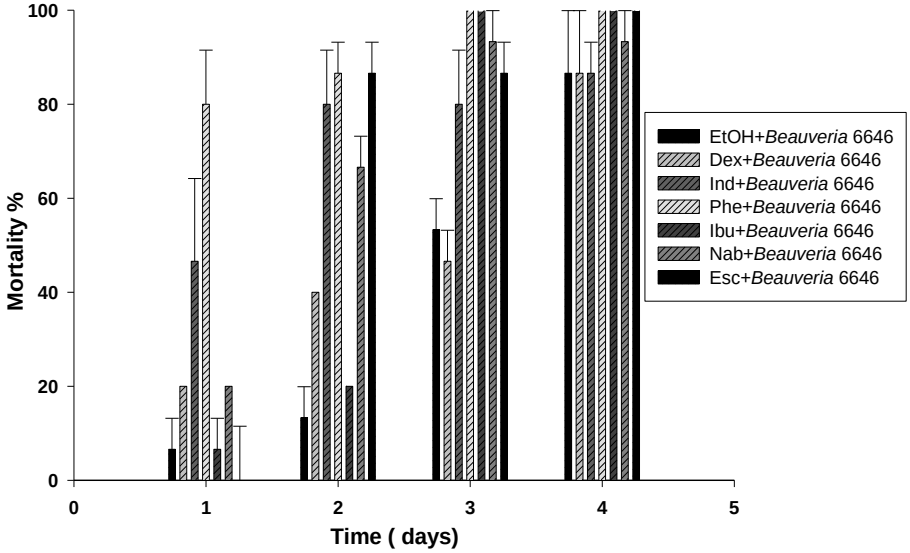

Figure 2. Effect of eicosanoid biosynthesis inhibitors on the mortality of *Spodoptera littoralis* larvae infected with of *Beauveria bassiana* (isolate 6646). Mortality was assessed at selected times after applications. The points indicate percentage mortality on days post-application.

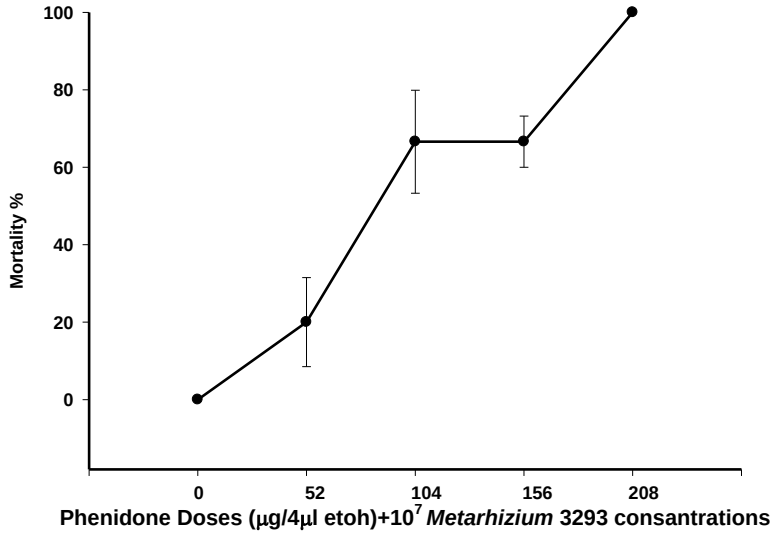

Figure 3. Influence of phenidone dosages on the mortality of *Spodoptera littoralis* larvae when co-injected with *Metarhizium anisoplia* (isolate 3293). Mortality was assessed after 24 hours. The points indicate percentage mortality after 24 hour post-applications.

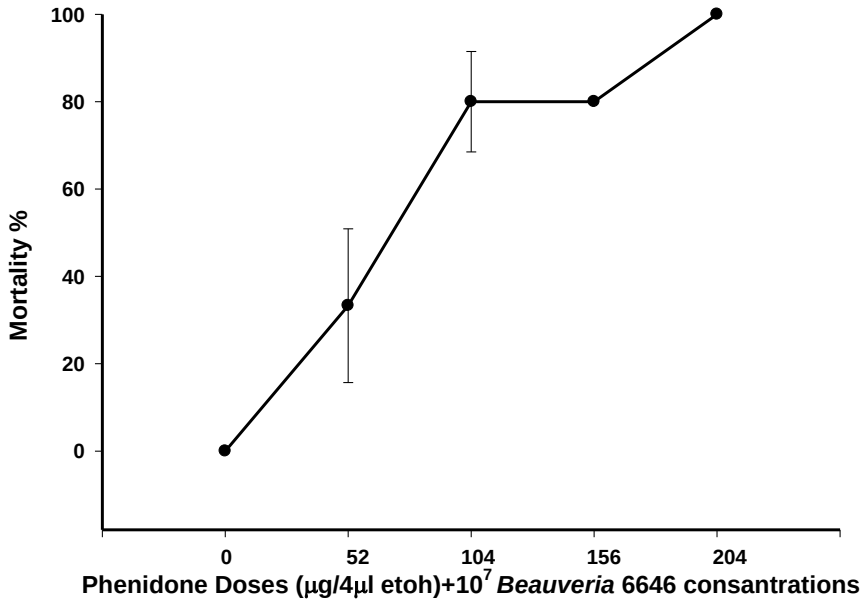

Figure 4. Influence of phenidone dosages on the mortality of *Spodoptera littoralis* larvae when co-injected with *Beauveria bassiana* (isolate 6646). Mortality was assessed after 24 hours. The points indicate percentage mortality after 24 hour post-application.

The idea that eicosanoids having role on insect cellular immunity was suggested by many researchers (Stanley, 2006; Stanley and Miller, 2006). There is now substantial evidence that eicosanoids are involved in insect-immune reactions against bacteria, fungi, protozoa, and parasitoid threats in a wide range of insects. The hypothesis that eicosanoids mediate nodulation reactions to fungal infection in *M. sexta* was tested (Dean et al., 2002; Lord et al., 2002). They indicated that eicosanoids mediate the cellular response of *Manduca sexta* against the fungal pathogens *B. bassiana* and *M. anisopliae*. Connick et al. (2001) have suggested that EBIs have synergistic effects with the bacterium, *S. marcescens* on the mortality of *Coptotermes formosanus.* Also Tunaz (2006) showed that when eicosanoid inhibitors were applied *P. brassicae* larvae with entomopathogenic fungus together, increased and faster mortality of *P. brassicae* larvae was seen. In addition, Tunaz and Küsek (2012; 2015) showed that

the mortality of *B. germanica* adults and *S. littoralis* larvae increased when the bacterium, *S. marcescens* co-administered with eicosanoid biosynthesis inhibitors to the insects. In parallel with these results, our results showed that the application of eicosanoid inhibitors with fungal agents to *S. littoralis* larvae increased the mortality of the insects.

As a result, we suggested that eicosanoid biosynthesis inhibitors have led to increased larval mortality of *S. littoralis* when co-applied with the fungal isolates and therefore microbial control programs can be enhanced.

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