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Towards eco-friendly pest control: synergistic potential of entomopathogenic fungi and plant essential oils against *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) and *Tenebrio molitor* Linnaeus, 1758 (Coleoptera: Tenebrionidae)

Ekolojik zararlı mücadelesinde alternatif stratejiler: *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) ve *Tenebrio molitor* (Linnaeus, 1758) (Coleoptera: Tenebrionidae)'a karşı entomopatojen funguslar ile bitkisel uçucu yağların etkileşimi

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ABSTRACT

Entomopathogenic fungi such as *Beauveria bassiana* and *Metarhizium brunneum* are important biological control agents, but their relatively slow action can limit efficiency. Essential oils, rich in bioactive compounds, represent potential complementary tools due to their insecticidal and repellent properties. The present study evaluated the individual and combined effects of *B. bassiana* (BMAUM-M6-4) and *M. brunneum* (V275) with thyme, rosemary, and eucalyptus essential oils (EOs) against *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) and *Tenebrio molitor* Linnaeus, 1758 (Coleoptera: Tenebrionidae) larvae. EOs were applied individually, simultaneously with fungal suspensions, or sequentially with a 24-hour interval to assess interactions. In addition, in vitro assays were conducted to determine the potential inhibitory effects of EOs on fungal mycelial growth and conidial germination.

The results revealed that thyme oil produced the strongest synergistic effect with both fungal isolates. Simultaneous application of thyme oil with *B. bassiana* or *M. brunneum* significantly increased larval mortality compared to single treatments. Rosemary oil showed moderate compatibility, although pre-exposure of larvae to rosemary reduced fungal efficacy. Eucalyptus oil contributed little to mortality under laboratory conditions. Importantly, in vitro assays showed that EOs at 1% concentration did not significantly inhibit fungal spore germination, and only minor reductions in mycelial growth were observed, particularly with eucalyptus oil showing the least effect.

Overall, combining entomopathogenic fungi with selected essential oils, particularly thyme and rosemary, enhanced pest mortality and demonstrated potential as an environmentally friendly alternative within integrated pest management strategies. Nevertheless, the effectiveness of these treatments depends on application sequence and host species, underscoring the need for optimization and validation under semi-field and field conditions.

INTRODUCTION

In the development of alternative pest control methods to replace chemical pesticides, the biological control agents used must be at least as effective as chemical pesticides while being environmentally safe, cost-effective, and free of harmful residues that may pose risks to humans and non-target organisms (Kumar et al. 2022). Within ecosystems, natural enemies of pests coexist; however, little is known about their interactions (Bardgett and van der Putten 2014). The main question of this study concerns how the simultaneous use of these pathogens may generate positive or negative results that influence the overall effectiveness of biological control applications. As a general principle, complex associations in nature tend to be more stable, and it has been hypothesized that biological control strategies employing mixtures of multiple antagonists will be more successful than those relying on a single pathogen (Lacey et al. 2001, van Lenteren et al. 2017).

Essential oils (EOs) are volatile and aromatic natural compounds obtained from different parts of plants (flowers, seeds, leaves, fruits, bark, etc.) through processes such as steam or water distillation, mechanical extraction, or dry distillation. They are typically liquid at room temperature, and range in color from colorless to pale yellow. Their use has been documented for thousands of years in traditional medicine, food preservation, and pest management. Particularly in recent years, growing concerns about environmental impacts have increased interest. Since they degrade rapidly in nature, EOs do not exhibit toxic effects in mammals and do not contribute to soil or water pollution. With their broad biological activity against pests, they possess insecticidal and repellent properties and can also influence pest growth and reproduction (Isman 2000, Isman et al. 2008). Numerous studies have reported insecticidal, ovicidal, repellent, attractant, and antifeedant effects of EOs against pest insects (Güdek and Çetin 2017, Kesdek et al. 2013, Kesdek et al. 2015, Kordali et al. 2006, Kordali et al. 2008, Regnault-Roger et al. 1993, Shaaya et al. 1993, Usanmaz Bozhüyük et al. 2018, Üstüner et al. 2018, Yıldırım et al. 2005).

Fungi represent the second largest group of soil-colonizing microorganisms after bacteria. Soil serves as an important reservoir for entomopathogenic fungi, and many species have been frequently isolated from it (Meyling and Eilenberg 2007). In agricultural ecosystems, the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae*, and *Paecilomyces lilacinus* are commonly encountered and exhibit broad host ranges (Klingen et al. 2002, Keller et al. 2003, Zimmermann 2007a, 2007b). Under natural conditions, these pathogens are responsible for frequent mortality within insect populations (Shah and Pell 2003). Unlike other entomopathogens, entomopathogenic fungi infect insects by penetrating the epicuticle through mechanical pressure or enzymatic activity (Ortiz-Urquiza and Keyhani 2013). For this reason, they play a particularly significant role in controlling insects with piercing-sucking mouthparts (Shah and Pell 2003).

In recent years increasing attention has been given to the combined use of entomopathogenic fungi and plant-derived EOs in integrated pest management strategies. Strong insecticidal and antibacterial properties of EOs, such as those obtained from thyme, clove, or fennel, can either enhance or interfere with the formation of fungal infection. Several studies have demonstrated that certain EOs can enhance fungal pathogenicity by increasing insect cuticle permeability or by promoting spore adhesion and penetration (Rocha et al., 2024; Lak et al., 2022). For instance, the co-application of *M. anisopliae* isolates with fennel or tarragon essential oils resulted in synergistic larvicidal activity against *Aedes aegypti*, significantly reducing lethal time values (Lak et al., 2022). Similarly, *Akanthomyces attenuatus* combined with botanical alkaloids such as matrine exhibited enhanced virulence and faster host mortality compared to individual applications (Wu et al., 2021). These findings highlight that the appropriate selection of EO type and concentration can play a key role in enhancing the overall efficacy of fungal-based biological control.

However, other research indicates that essential oils may also exhibit antagonistic effects on fungal entomopathogens, depending on their chemical composition and concentration. Oils rich in phenolic compounds such as thymol, eugenol, and carvacrol can disrupt fungal cell membranes and inhibit spore germination, thereby reducing fungal viability and persistence (Bitencourt et al., 2022). Some combinations of *B. bassiana* and *M. anisopliae* with clove, thyme oils or garlic were shown to suppress fungal sporulation or delay host infection (Bruck, 2005, Eckard et al. 2017; Rocha et al., 2024). Consequently, the interactions between entomopathogenic fungi and essential oils can range from synergistic to antagonistic, underscoring the need for comprehensive compatibility testing prior to field applications.

In this study, the effects of selected plant essential oils (EOs) and entomopathogenic fungi were investigated on the greater wax moth, *Galleria mellonella* L. (Linnaeus, 1758) (Lepidoptera: Pyralidae), and the mealworm, *Tenebrio molitor* Linnaeus, 1758 (Coleoptera: Tenebrionidae), under laboratory conditions. The independent effects of plant EOs and entomopathogenic fungi on these pests were examined, their combined applications were tested to evaluate the insecticidal efficacy of EOs and entomopathogenic fungi when applied at low concentrations. Furthermore, the in-vitro effects of EOs on the mycelial growth and spore

germination of entomopathogenic fungi in culture media were assessed. The overall aim of this study is to achieve effective pest control through the combined application of EOs and entomopathogenic fungi, optimizing dosages to maximize insect mortality while minimizing environmental impact

MATERIALS AND METHODS

Fungal isolates

The entomopathogenic fungus *B. bassiana* (BMAUM-M6-4) isolate used in this study was obtained from soil samples collected in Isparta Province using the Galleria bait method. *Metarhizium brunneum* (V275), originally isolated from *Cydia pomonella* and supplied by the Biocontrol and Natural Products (BANP) Group at Swansea University (UK), was also employed. For efficacy trials, *M. brunneum* (V275) and EOs were tested against the mealworm, *T. molitor*, whereas *B. bassiana* (BMAUM-M6-4) and EOs were tested against the greater wax moth, *G. mellonella* larvae. The rosemary, eucalyptus, and thyme EOs used in the assays were obtained from Botalife® (Isparta, Türkiye).

Insect rearing

Tenebrio molitor larvae were maintained on a basal substrate composed of rye flour and potato slices. The larvae were kept in ventilated containers and provided with fresh substrate and potatoes weekly to ensure adequate nutrition and moisture (Rui et al. 2018). Larvae of *G. mellonella* were reared using beeswax harvested from old honeycombs, mimicking their natural diet of wax, brood remains and pollen. The wax substrate was replaced or supplemented as needed to ensure continuous access to fresh material (Birah et al. 2008). Both insect colonies were cultured under controlled laboratory conditions at 27± 2°C and 60 ± 5% relative humidity (RH), with a photoperiod of 12 h:12 h light: dark and constant monitoring of substrate cleanliness and larval health. Ventilation and hygienic practices were strictly followed prevent mold growth and contamination of the rearing containers.

Preparation of fungal suspensions

The fungal isolates were cultured on Potato Dextrose Agar (PDA) at 25 ± 2°C in the dark. After 10–17 days of growth, conidia were harvested into 20 of mL sterile distilled water containing 0.1% Tween 20. Conidial suspensions were quantified using a hemocytometer, and adjusted to the required final concentrations for bioassays (Inglis et al. 2012).

Bioassays with essential oils

Third-instar larvae of *G. mellonella* and *T. molitor* were starved for two hours prior to treatments. Rosemary, eucalyptus, and thyme essential oils (EOs) were prepared at concentrations of 0.1%, 1%, and 3%, and larvae were dipped in 50 mL of the prepared solution for 5 seconds. Sterile distilled water served as the control. Larval mortality was recorded daily for seven days, and dead larvae were removed from Petri dishes. Each treatment consisted of six larvae and was replicated five times, resulting in a total of 30 larvae per treatment. All treatments were repeated twice on separate occasions. Mortality data were corrected using Abbott's formula, and percentage mortality was calculated (Abbott 1925).

Bioassays with entomopathogenic fungi

To determine the lethal concentration for 50% mortality (LC_{50}), third-instar larvae of *G. mellonella* and *T. molitor* were immersed for 5 seconds in 10 mL suspensions containing 10^4, 10^5, 10^6, 10^7, or 10^8 conidia/ mL. Larvae were then transferred to 9-cm Petri dishes lined with moist filter paper (five larvae per dish). The control group received 0.3% Tween 80. Each treatment was replicated six times and repeated twice on different days. Larval mortality was recorded daily for 10 days. Dead larvae were surface-sterilized with 2% sodium hypochlorite, 70% ethanol, and sterile water before being placed in Petri dishes with moist filter paper to confirm fungal outgrowth (Gołębiowski et al. 2021). Mortality data were corrected using Abbott's formula, and LC_{50} values were estimated using Probit analysis (Abbott 1925).

Combined applications of entomopathogenic fungi and essential oils

Based on the LC_{50} values, concentrations of 10^5 conidia/mL for *B. bassiana* and 10^6 conidia/ mL for *M. brunneum* (Table 1), together with 1% concentrations of essential oils, were selected for combination treatments, which were applied using the dipping method. In simultaneous treatments, larvae were dipped 5 seconds in EOs solutions, placed on sterile filter paper for 10 min to dry, and then exposed to fungal suspensions. In the sequential treatment experiments, two exposure sequences were tested. In the first sequence, larvae were treated with EOs, placed in Petri dishes with food, and incubated for 24 hours before inoculation with fungal conidia. In the second sequence, larvae were first exposed to the fungal suspension, followed by incubation for 24h, after which EOs were applied. Each treatment involved six larvae with five replicates and was repeated twice. Treatments involving only the application of entomopathogenic fungi or only essential oils (EOs) served as negative controls, while the application of distilled water (dH_2O) was designated as the positive control. Mortality was monitored daily for 6 to 7 days, and cadavers were incubated in moist chambers to confirm fungal development (Gołębiowski et al. 2021).

Experiments were conducted at 25 ± 2°C and 60 ± 5% RH. Mortality data were corrected with using Abbott's formula. One-way ANOVA followed by Tukey's test was performed to compare treatment means. Data analysis was conducted using SPSS version 17.0 (SPSS Inc., Chicago, IL) (Abbott 1925).

In-vitro effects of essential oils on fungal growth

For mycelial growth assays, 5-mm mycelial discs from 7–10 day-old fungal cultures were placed onto PDA plates. EOs at 1% concentration as used in bioassays were spread onto the surface of PDA plates (1 mL per plate) using a Drigalski spatula. Control plates received sterile distilled water. Plates were sealed with Parafilm and incubated at 25 ± 2°C in the dark at 75–80% relative humidity (RH) (Soylu et al. 2006). Colony diameters were measured daily, and growth inhibition was calculated using Abbott's formula (Abbott 1925, Soylu et al. 2006, Soylu et al. 2007).

In-vitro effects of essential oils on spore germination

For conidial germination assays, PDA plates (9 cm diameter) were treated with 100 µL of 1% essential oil (EO) solution spread using a Drigalski spatula. A 100 µL conidial suspension (1 × 10^5 conidia/ mL) was then spread over the EO-treated surface. Plates were incubated at 24 ± 1°C. Controls received sterile distilled water. Each treatment was replicated three times. After 24h of incubation, 200–300 conidia were counted from two different fields per plate to assess germination rates (Soylu et al. 2007).

RESULTS

Effects of essential oils on Tenebrio molitor (L.) and Galleria mellonella (L.) larvae

In third-instar larvae of *G. mellonella*, the 3% concentrations of eucalyptus and thyme EOs produced significantly higher mortality compared to the control and other treatments, reaching 50.2% and 48.2% mortality, respectively, by days five and six respectively. In contrast, rosemary oil at 1% caused the lowest mortality (0.5%), while the control group exhibited 3% mortality throughout the trial. None of the EOs produced statistically significant differences from the control at the lowest concentration tested (0.1%) (Table 1).

For *T. molitor* larvae, a clear dose-dependent response was observed, particularly with thyme oil, where the 3% concentration resulted in 100% mortality, whereas 1% caused only 13.8%. Rosemary oil exhibited intermediate activity, with mortality rates of 17.2% and 27.6% at 1% and 3%, respectively. Eucalyptus oil showed the weakest effect, producing 6.9% mortality at 1% and 20.7% at 3%. Control mortality in *T. molitor* remained approximately 3% throughout the experiment (Table 1). These results indicate that thyme essential oil has the highest insecticidal potential, especially against *T. molitor*, and highlight the concentration-dependent nature of essential oil efficacy.

Effects of entomopathogenic fungi on larvae

The bioassay results demonstrated that increasing conidial concentrations of the entomopathogenic fungi led to progressively higher larval mortality. For *B. bassiana* against *G. mellonella* larvae, mortality rates reached 51.2%, 59.7%, 70.4%, and 92.6% at 1×10^5, 1×10^6, 1×10^7, and 1×10^8 spores/mL, respectively, after six days of exposure, whereas the control group showed only 5% mortality. Similarly, *M. brunneum* caused progressive larval mortality in *T. molitor*. At day six, mortality ranged from 38.5% at 1×10^5 spores/mL to 69.2% at 1×10^8 spores/ mL, while the control group exhibited 10% mortality (Table 2). Probit analysis of the sixth-day data showed that *B. bassiana* was capable of achieving both LC_{50} and LC_{90} values within the tested concentration range, indicating a strong pathogenic potential against the target larvae (Table 3). The LC_{50} value of the *B. bassiana* isolate for *G. mellonella* larvae was found to be ≈ 1.67×10^5 spores/ mL. In contrast, for *M. brunneum*, although an LC_{50} value could be determined, the maximum mortality did not reach 90%, and therefore an LC_{90} value could not be estimated under the experimental conditions.

Table 1. Mortality rates of third instar larvae of *Galleria mellonella* and *Tenebrio molitor* after 6th day exposure to different concentrations of essential oils under laboratory conditions (%mortality±SE)

Test insects	Essential Oils (6th day)						Control[a]
	Thyme[a]		Rosemary[a]		Eucalyptus[a]		
	1%	3%	1%	3%	1%	3%	dH_2O
G. mellonella	44.4±0.7a	48.2±0.8a	0.5±0.3c	18.5±0.4b	48.2±0.8a	50.2±0.5a	3±0.2c
T. molitor	13.8±0.3bc	100±0.0a	17.2±0.3bc	27.6±0.4b	6.9±0.3cd	20.7±0.5bc	3±0.2d

[a] Values represent mean ± SE. Different letters within the same lane indicate significant differences according to Tukey's test ($p<0.05$).

The LC_{50} value of the *M. brunneum* isolate for *T. molitor* larvae was found to be ≈ 1.44×106 spores/ mL. These results emphasize the importance of concentration-dependent virulence in entomopathogenic fungi and highlight the variability in their efficacy depending on the host–pathogen interaction.

Table 2. Mortality rates of *Galleria mellonella* and *Tenebrio molitor* larvae exposed to different concentrations of entomopathogenic fungi (%mortality±SE)

Concentration (spores/mL)	Mortality % (Day 6)	
	***B. bassiana* (BMAUM-M6-4)**	***M. brunneum* (V275)**
	***G. mellonella*[a]**	***T. molitor*[a]**
$1x10^5$	51.2±0.3 c	38.5±0.6 a
$1x10^6$	59.7±0.2 cb	50.0±0.8 a
$1x10^7$	70.4±0.3 b	65.4±0.7 a
$1x10^8$	92.6±0.2 a	69.2±0.7 a
Control (dH_2O+Tween)	10.0±0.2 d	10.0±0.2 b

[a] Values represent mean ± SE. Different letters within the same lane indicate significant differences according to Tukey's test ($p < 0.05$).

oils yielded mortality rates exceeding 80% by day six, which were statistically distinct from their respective controls ($p<0.05$, Tukey test). Eucalyptus oil, although less potent as a single treatment, also enhanced fungal pathogenicity when applied in combination, with increasing mortality exceeding 60% (Table 4).

The combined application of *B. bassiana* and essential oils (EOs; thyme, rosemary, and eucalyptus) produced distinct mortality patterns in *G. mellonella* larvae depending on both the type of oil and the mode of application (Table 4). In the control group treated with distilled water (dH_2O), mortality remained approximately 6% throughout the experiment. *B. bassiana* or individual EOs alone caused relatively moderate mortality rates during the six-day observation period. In general, simultaneous treatments of the fungal isolate with EOs resulted in markedly higher mortality rates than either single applications or sequential treatments, suggesting a possible synergistic interaction. For instance, the combination of B. bassiana with thyme oil induced rapid larval mortality, reaching over 80% by day 5 and exceeding 88% by day 6, whereas thyme oil or the fungus alone caused significantly lower mortality at the same time points ($p<0.05$, Tukey test). A similar result was observed with rosemary oil, where simultaneous application with *B. bassiana* resulted in nearly 90% mortality by the sixth day, in contrast to less than 52% for the fungus alone and negligible mortality for rosemary oil alone. In the case of eucalyptus

Table 3. Estimated LC_{50} and LC_{90} values of entomopathogenic fungi against insect larvae (6th day mortality, Probit analysis)

Fungal Isolate	Host Insect	LC_{50} (spores/mL)	Log_{10} (LC_{50})	LC_{90} (spores/mL)	Log_{10} (LC_{90})
B. bassiana (BMAUM-M6-4)	*G. mellonella*	1.67×10^5	5.22	8.12×10^7	7.91
M. brunneum (V275)	*T. molitor*	1.44×10^6	6.16	ND*	ND*

[a] ND = Not determined, since maximum mortality did not exceed 70% under tested conditions.

Combination application of entomopathogenic fungi and essential oils

The bioassay results demonstrated that the combined application of *B. bassiana* with EOs significantly increased larval mortality of *G. mellonella* compared to single treatments. Control group treated with distilled water (dH_2O) remained approximately 6% throughout the experiment. While *B. bassiana* or individual EOs alone caused relatively moderate mortality rates during the six-day observation period, their simultaneous or sequential applications produced markedly higher effects (Table 4). In particular, the combinations with rosemary and thyme oil, the synergistic effect was less pronounced although combined treatments enhanced larval mortality compared to individual applications, the maximum mortality did not exceed 63% by day 6. Interestingly, the sequential application (oil followed by fungus, or fungus followed by oil after 24h) generally produced intermediate effects, often lower than simultaneous treatments (Table 4).

Similar results to those observed in the *B. bassiana* and EOs experiments were also observed in *M. brunneum* and EOs. Treatments with thyme oil exhibited the strongest synergistic effects when combined with *M. brunneum* (Table 5). Control mortality in *T. molitor* remained approximately

Table 4. Mortality rates (%) of *Galleria mellonella* larvae treated with *Beauveria bassiana* (BMAUM-M6-4) isolate and essential oils (thyme, rosemary, eucalyptus) applied individually, simultaneously, or sequentially (24 h interval)

Day	B.b.*	Thyme[a]				Rosemary[a]				Eucalyptus[a]			
		Oil	B.b.+Oil*	Oil→B.b. (24h)*	B.b.→Oil (24h)*	Oil	B.b.+Oil*	Oil→B.b. (24h)*	B.b.→Oil (24h)*	Oil	B.b.+Oil*	Oil→B.b. (24h)*	B.b.→Oil (24h)*
1	0.0±0.0 b	10.0±0.3 b	63.3±0.7 a	0.0±0.0 b	0.0±0.0 b	0.3±1.2 b	3.3±1.2 b	0.0±0.0 a	3.3±1.2 b	6.7±0.2 ba	20.0±0.5 a	0.0±0.0 b	0.0±0.0 b
2	0.0±0.0 b	16.7±0.7 b	70.0±0.6 a	0.0±0.0 b	0.0±0.0 b	0.7±0.2 b	10.0±0.5 b	0.0±0.0 b	3.3±1.2 b	13.3±0.2 ba	23.3±0.5 b	0.0±0.0 b	0.0±0.0 b
3	6.6±0.2 b	16.7±0.7 b	73.3±0.5 a	3.3±0.2 b	23.3±0.2 b	0.7±0.2 b	30.0±0.4 a	3.3±0.4 b	26.7±0.2 a	20.0±0.5 ba	33.3±0.7 a	3.3±0.3 b	16.7±0.2 ba
4	13.3±0.2 b	16.7±0.7 b	73.3±0.5 a	3.3±0.2 b	53.3±0.3 a	0.7±0.2 b	50.0±0.3 a	3.3±1.2 b	63.3±0.3 a	20.0±0.5 a	36.7±0.3 a	3.3±0.3 b	33.3±0.7 a
5	26.7±0.2 b	20.0±0.6 b	80.0±0.4 a	7.1±0.3 b	71.4±0.5 a	1.0±0.2 b	73.3±0.5 a	3.4±0.2 b	85.7±0.3 a	26.7±0.2 b	50.0±0.7 ab	3.6±0.3 c	57.1±0.7 a
6	51.9±0.3 b	44.4±0.7 b	81.5±0.3 a	40.7±0.3 b	88.9±0.3 a	0.5±0.4 c	88.9±0.2 a	18.5±0.4 b	88.9±0.2 a	48.2±0.8 a	55.6±0.5 a	29.6±0.4 a	63.0±0.7 a

[a] Values represent mean ± SE. Different letters within the same row indicate significant differences according to Tukey's test ($p < 0.05$).
* Meaning of abbreviations, B.b.: *B. bassiana*, B.b.+Oil: same time application, Oil→B.b. (24): *B. bassiana* application 24 hours after essential oil application, B.b.→Oil (24h): essential oil application 24 hours after *B. bassiana* application

10% throughout the experiment. Simultaneous or sequential applications (24h apart) consistently yielded high mortality levels, exceeding 70% by day 7, compared to only 14% mortality for the fungal isolate alone. This indicates a clear enhancement of fungal pathogenicity by thyme oil, suggesting a synergistic interaction. Rosemary oil produced more moderate effects. When applied individually, larval mortality remained below 20%, while its combination with *M. brunneum* increased mortality to 30–46% depending on the application strategy. Although the effect was statistically significant compared to controls, the enhancement was less obvious than with thyme, pointing to a weaker additive rather than strongly synergistic interaction. Eucalyptus oil alone had minimal larvicidal activity (<10% mortality by day 7). However, its combination with *M. brunneum* produced variable results. While simultaneous application resulted in modest increases in mortality (37%), the sequential treatment (oil followed by fungus after 24h) showed a marked improvement, reaching 70% mortality at day 7. This suggests that temporal separation between treatments can optimize fungal infection efficacy, likely by reducing potential antagonism at the cuticular level.

Table 5. Mortality rates (%) of *Tenebrio molitor* larvae treated with *Metarhizium brunneum* (V275) isolate and essential oils (thyme, rosemary, eucalyptus) applied individually, simultaneously, or sequentially (24 h interval)

Day	M.b.*	Thyme[a]				Rosemary[a]				Eucalyptus[a]			
		Oil	B.b.+Oil*	Oil→B.b. (24h)*	B.b.→Oil (24h)*	Oil	B.b.+Oil*	Oil→B.b. (24h)*	B.b.→Oil (24h)*	Oil	B.b.+Oil*	Oil→B.b. (24h)*	B.b.→Oil (24h)*
1	0.0±0.0 b	6.7±0.2 ab	20.0±0.5 a	23.3±0.7 a	0.0±0.0 b	0.0±0.0 b	3.3±0.2 b	0.0±0.0 b	0.0±0.0 b	0.0±0.0 b	0.0±0.0 b	0.0±0.0 b	0.0±0.0 b
2	0.0±0.0 c	10.0±0.3 b	23.3±0.6 a	33.3±0.6 a	6.7±0.4 b	0.0±0.0 c	10.0±0.2 b	0.0±0.0 c	6.7±0.2 b	3.3±0.2 c	6.7±0.2 b	0.0±0.0 c	3.3±0.2 c
3	0.0±0.0 c	10.3±0.3 b	34.0±0.4 a	40.7±0.4 a	27.3±0.4 ab	0.0±0.0 c	10.0±0.2 b	10.0±0.6 b	6.7±0.2 b	0.0±0.0 c	13.3±0.4 b	0.0±0.0 c	3.3±0.2 c
4	3.3±0.2 c	6.9±0.3 c	50.7±0.4 a	65.3±0.6 a	62.0±0.8 a	3.4±0.2 c	37.3±0.5 a	13.3±0.8 bc	28.7±0.6 b	3.3±0.2 c	23.3±0.4 b	53.3±1.0 a	6.7±0.4 c
5	3.3±0.2 c	10.3±0.3 bc	63.3±0.6 a	68.0±0.7 a	62.0±0.8 a	17.2±0.3 bc	36.0±0.5 b	13.3±0.8 bc	32.0±0.7 b	6.9±0.3 c	37.3±0.5 b	56.7±1.1 a	6.7±0.4 c
6	10.0±0.4 c	13.8±0.3 c	66.7±0.7 a	68.0±0.7 a	70.0±0.5 a	17.2±0.3 c	39.3±0.6 b	13.3±0.8 c	39.3±1.0 b	6.9±0.3 c	37.3±0.5 b	60.0±1.2 a	18.0±0.6 bc
7	14.1±0.6 c	13.8±0.3 c	66.7±0.7 a	72.0±0.8 a	77.3±0.4 a	17.2±0.3 c	46.0±0.6 b	13.3±0.8 c	39.3±1.0 b	6.9±0.3 c	37.3±0.5 b	70.0±0.9 a	18.0±0.6 bc

[a] Values represent mean ± SE. Different letters within the same row indicate significant differences according to Tukey's test ($p < 0.05$).
* Meaning of abbreviations: M.b.: *M. brunneum*, B.b.+Oil: same time application, Oil→M.b. (24): *M. brunneum* application 24 hours after essential oil application, M.b.→Oil (24h): essential oil application 24 hours after *M. brunneum* application

Effects of essential oils on the in vitro development of entomopathogenic fungi

The contact effects of EOs on the mycelial growth of *B. bassiana* (BMAUM-M6-4) and *M. brunneum* (V275) are presented in Table 6. At a concentration of 1%, EOs exhibited only limited inhibition of fungal mycelial growth, with values not exceeding 22.2%. Among the tested oils, eucalyptus showed the least inhibitory effect, particularly on *M. brunneum*, where inhibition was as low as 2.8%. In contrast, thyme and rosemary oils caused relatively higher, yet still moderate, reductions in mycelial growth No significant reduction in germination rates was observed across treatments, with values remaining above 92% in all cases. Notably, germination rates for *B. bassiana* ranged between 95.3–98.0%, while those for *M. brunneum* remained between 92.3–97.7%, comparable to the control (Table 6). These results indicate that EOs at sublethal concentrations do not compromise the fundamental reproductive ability of the fungi.

Table 6. Effects of essential oils on mycelial growth and spore germination (%) (n=200 spores) of *Beauveria bassiana* (BMAUM M6-4) and *Metarhizium brunneum* (V275), and mycelial growth inhibition percentage (±SD)

Oils	Fungal Isolates	Mycelial growth inhibition (%)[a]	Spore germination (%)[a]
Thyme	*Beauveria bassiana*	22.2±0.09 b	95.3±1.1 a
	Metarhizium brunneum	13.6±0.13 b	97.7±0.2 a
Rosemary	*B. bassiana*	18.6± 0.08 b	98.0±0.4 a
	M. brunneum	19.5±0.14 b	92.3±0.5 a
Eucalyptus	*B. bassiana*	12.7± 0.08 b	96.3± 0.8 a
	M. brunneum	2.8±0.03 a	97.0±0.6 a
dH_2O	*B. bassiana*	-	98.3±0.8 a
	M. brunneum	-	100.0±0.0 a

[a] Values represent mean ± SE. Different letters within the same column indicate significant differences according to Tukey's test ($p < 0.05$).

DISCUSSION

Among microbial control agents used against insect pests, entomopathogenic fungi stand out as some of the most promising alternatives to conventional pesticides. Their conidia adhere to the insect cuticle, penetrate the host integument internally, and proliferate internally (Boucias and Pendland 1982, Butt 2002). These fungi are preferred as microbial insecticides because they do not leave toxic residues on agricultural products and are considered safe for non-target organisms, including beneficial insects. Moreover, they are regarded as environmentally friendly and safe for humans and animals. As they do not harm natural enemies, entomopathogenic fungi have potential in integrated pest management (IPM) strategies (Inglis et al. 2001). However, they generally act more slowly compared to chemical insecticides, which limits their use as individual control agents (Butt et al. 2016). Therefore, combining them with other environmentally benign control methods may enhance their effectiveness.

Plant-derived EOs, obtained from various aromatic plants, are rich in bioactive compounds such as terpenoids, alkaloids, and flavonoids, many of which possess insecticidal and repellent properties. Several studies have demonstrated their broad biological activities, including insecticidal, repellent, growth-regulating, and reproductive-inhibitory effects (Arnason et al. 1989, Hedin et al. 1997, Isman, 2000, Isman et al. 2008). Approximately 2.000 plant species are known to produce EOs with insecticidal or repellent activity. Numerous reports further confirm that these oils and their constituents exhibit toxic, deterrent, and antifeedant properties, making them promising candidates for environmentally friendly pest management (Çetin et al. 2006, Erdoğan and Toros 2005, Kanat and Alma 2004, Kesdek et al. 2014, Kordali et al. 2006, Kordali et al. 2008, Shaaya et al. 1993, Türkölmez and Soylu 2014).

To overcome the limitations of using a single biocontrol agent, research has increasingly focused on combining entomopathogens with other biological or botanical agents. Since EOs have demonstrated broad-spectrum antifungal activity (Batish et al. 2008, Usanmaz Bozhüyük and Kordali 2019, Kalemba and Kunicka 2003, Koul et al. 2008, Soković and van Griensven 2006), it is crucial to assess their compatibility with entomopathogenic fungi

before considering them for integrated use. In this study, the effects of thyme, rosemary, and eucalyptus EOs (at 1% concentration) were evaluated on the mycelial growth and spore germination of *B. bassiana* (BMAUM-M6-4) and *M. brunneum* (V275). Overall, the in vitro assays demonstrated that low concentrations of thyme, rosemary, and eucalyptus oils exert only minor inhibitory effects on fungal mycelial growth and have no detrimental impact on spore germination. A previous study investigated the interaction between *M. brunneum*, garlic extract (*Allium sativum* L., Amaryllidaceae), and wireworms, *Agriotes obscurus* (L.), (Coleoptera: Elateridae) as the target hosts. The results demonstrated antagonism in the combined treatment, likely because the garlic extract reduced the rate of spore germination (Eckard et al. 2017). These findings are important from an integrated pest management (IPM) perspective, as they suggest that selecting an appropriate EO type and concentration can significantly improve the overall effectiveness of fungal-based biological control. When applied against *G. mellonella* larvae, the simultaneous application of thyme oil and *B. bassiana* (B.b.+Thyme) produced significantly higher mortality from the first day, compared to single treatments. Similarly, when thyme was applied 24 hours after *B. bassiana* [B.b.+Thyme (24)], mortality remained high and similar to the simultaneous application, indicating a synergistic interaction. In contrast, when thyme was applied 24 hours before the fungus [Thyme+B.b.(24)], fungal pathogenicity was strongly reduced, and mortality rates were comparable to thyme alone, suggesting an antagonistic effect. Interestingly, similar synergistic interactions were observed with *M. brunneum* on *T. molitor* larvae, where combined applications resulted in significantly higher mortality than single treatments, particularly from the early stages of infection. These findings highlight the potential of thyme oil–fungus combinations as effective biocontrol strategies, which is consistent with previous studies reporting strong insecticidal activity of *Origanum* spp. oils in a dose-dependent manner (Ayvaz et al. 2010, Bedini et al. 2021, Benelli et al. 2019, Khalfi et al. 2008, Mudrončeková et al. 2019, Toshova et al. 2022). However, the commercial viability of EOs is limited by their low yield (1–3%) and high production cost (Çakır 1992, Chakravarty et al. 2023, Hongthong et al. 2025). If cost obstacles can be overcome, thyme oil could be an effective component of IPM programs (Rozman et al. 2007, Tunç et al. 2000).

In the case of rosemary oil, simultaneous application with *B. bassiana* or its application after fungal treatment resulted in relatively higher mortality compared to single treatments, whereas applying rosemary prior to fungal exposure significantly reduced fungal virulence. Against *T. molitor* larvae, rosemary oil combined with *M. brunneum* did not exhibit synergistic effects, with mortality levels of approximately 46%. Rosemary oil, widely used as a repellent, fumigant, and contact insecticide (Hori 1998, Koschier and Sedy 2003, Papachristos et al. 2004), is known for its variability in chemical composition depending on genotype, growth stage, and processing methods (Isman 2006). Such variability may explain the limited synergistic effects observed in our experiments.

Eucalyptus oil, when combined with *B. bassiana* or *M. brunneum*, did not significantly enhance or inhibit larval mortality, suggesting a largely neutral interaction. Although eucalyptus oil has been extensively reported as a natural repellent, antifeedant, and antiseptic (Brooker and Kleinig 2016, Erler et al. 2006, Lucia et al. 2007, Rajendran and Sriranjini 2008, Tinkeu et al. 2004), its repellent activity may not translate into larvicidal effects under our contact exposure conditions. Thus, future studies should focus on evaluating its repellent potential rather than direct toxicity when combined with entomopathogens.

Overall, the interaction between EOs and entomopathogenic fungi varies from synergistic to antagonistic depending on the order of application, fungal species, and oil type. Several studies have addressed such interactions. Razmjou et al. (2016) reported increased mortality of *Aphis gossypii* when combining *Lecanicillium muscarium* with plant-derived oils, confirming the potential of such strategies. Based on the study that evaluated the insecticidal activity, as well as the synergic effects of EOs extracted from fennel (*Foeniculum vulgare*), tarragon (*Artemisia dracunculus*), and lavender (*Lavandula angustifolia*), together with and three isolates of an entomopathogenic fungus *M. anisopliae* against the bean weevil, *Acanthoscelides obtectus adults*, the treatment resulted in synergistic effects and amplified insect mortality (Lak et al. 2022). The combination of *M. anisopliae* and *Schinus molle* L. EO exhibited a synergistic effect in targeting *Ae. aegypt*i larvae, whereas *B. bassiana*, in combination with EO, had an antagonistic effect (Bitencourt et al. 2022). The results of another study, which likewise targeted *Ae. aegypti* larvae with a combination of *Illicium verum* EO with *M. anisopliae* and *B. bassiana*, were comparable; however, the effect on *B. bassiana* was additive rather than antagonistic (Rocha et al. 2024). In our study, thyme and rosemary oils showed promise for integration with entomopathogens at sublethal concentrations, whereas eucalyptus oil was less effective.

The overarching goal of integrated pest management is to suppress pest populations below the economic threshold by combining multiple control agents. However, identifying whether these combinations are synergistic, antagonistic, or neutral is crucial for successful implementation. Based

on our findings, low concentrations of thyme and rosemary oils may be compatible with entomopathogenic fungi and could enhance their efficacy in insect pest management. Future research should broaden these trials across different fungal isolates, essential oil chemotypes, and pest species and should be conducted under laboratory, semi-field, and field conditions to establish robust and cost-effective IPM strategies.

In summary, this study demonstrates that the combination of entomopathogenic fungi (*B. bassiana* and *M. brunneum*) with selected EOs can significantly influence larval mortality in target pests, with outcomes ranging from synergistic to antagonistic depending on the oil type and application sequence. Thyme oil, in particular, enhanced fungal pathogenicity when applied simultaneously or after fungal exposure, indicating its strong potential for integration into pest management strategies. Rosemary oil exhibited moderate compatibility, while eucalyptus oil showed limited larvicidal contribution under contact exposure. Importantly, at sublethal concentrations, none of the oils inhibited fungal growth or spore germination, supporting their potential compatibility in combined use. Overall, these findings highlight the promise of integrating entomopathogenic fungi with plant-derived EOs as an eco-friendly approach to pest control. Further research under semi-field and field conditions is essential to optimize these combinations and evaluate their practical applicability within integrated pest management programs.

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ÖZET

Beauveria bassiana ve *Metarhizium brunneum* gibi entomopatojen funguslar önemli biyolojik mücadele etmenleri arasında yer almakla birlikte, nispeten yavaş etki göstermeleri etkinliklerini sınırlayabilmektedir. Biyoaktif bileşenlerce zengin olan bitkisel uçucu yağlar, insektisit ve kovucu özellikleri nedeniyle potansiyel tamamlayıcı ajanlar olarak değerlendirilmektedir. Bu çalışmada, *B. bassiana* (BMAUM-M6-4) ve *M. brunneum* (V275) izolatlarının kekik, biberiye ve okaliptüs uçucu yağları ile *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) ve *Tenebrio molitor* Linnaeus, 1758 (Coleoptera: Tenebrionidae) larvalarına karşı tekli ve kombine etkileri incelenmiştir. Uçucu yağlar tek başına, fungal süspansiyonlarla eş zamanlı veya 24 saat aralıkla uygulanarak etkileşimleri değerlendirilmiştir. Ayrıca, uçucu yağların fungusların miselyum gelişimi ve konidiyal çimlenmesi üzerindeki olası engelleyici etkilerini belirlemek için in-vitro testler gerçekleştirilmiştir.

Sonuçlar, kekik yağının her iki fungus izolatı ile en güçlü sinerjistik etkiyi oluşturduğunu göstermiştir. Kekik yağı ile *B. bassiana* veya *M. brunneum*'un eş zamanlı uygulanması, tekli uygulamalara kıyasla larva ölüm oranını anlamlı derecede artırmıştır. Biberiye yağı orta düzeyde uyumluluk göstermiş ancak larvaların biberiye ile ön işlem görmesi durumunda fungusun etkinliği azalmıştır. Okaliptüs yağı ise ölüm oranına sınırlı katkı sağlamıştır. Önemli olarak, in-vitro testler uçucu yağların %1 konsantrasyonda fungal spor çimlenmesini anlamlı şekilde engellemediğini, miselyum gelişiminde ise yalnızca küçük düşüşler gözlendiğini ve bu etkiler arasında en düşük etkinin okaliptüs yağında ortaya çıktığını göstermiştir.

Genel olarak, özellikle kekik ve biberiye yağlarının entomopatojen funguslarla kombinasyonu zararlı ölüm oranını artırmış ve entegre zararlı yönetimi stratejileri kapsamında çevre dostu bir alternatif olarak potansiyel göstermiştir. Bununla birlikte, uygulama sırası ve konukçu türüne bağlı olarak etkinlik değişmekte olup, yarı-tarla ve tarla koşullarında optimizasyon ve doğrulamaya ihtiyaç duyulmaktadır.

Anahtar kelimeler: *Beauveria bassiana*, *Metarhizium brunneum*, uçucu yağlar, biyolojik mücadele, etkileşim

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