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ORIGINAL ARTICLE

Entomopathogenic fungi and plant essential oils are not compatible in controlling *Tribolium castaneum* (Herbst)

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Abstract

Entomopathogenic fungi (EPF) and essential oils (EOs) can show either positive or negative interactions when used for controlling insect pests. First, the insecticidal efficacy of EPF including *Beauveria bassiana* isolates Z1 and IRAN1395C, *Lecanicillium lecanii*, and *Paecilomyces lilacinus* was tested against adults of *Tribolium castaneum* using two methods (standard insect dip and wheat diet incorporation). Additionally, the toxicity of EOs from *Trachyspermum ammi*, *Foeniculum vulgare*, *Eucalyptus globulus*, *Salvia mirzayanii*, *Majorana hortensis*, and *Thymus vulgaris* was evaluated against adult *T. castaneum*. Thereafter, the effect of an LC_{25} concentration of *F. vulgare* (86.13 µl L^{-1}), *T. ammi* (235.2 µl L^{-1}), and *E. globulus* (111.33 µl L^{-1}) EOs on mycelial growth, spore germination, and sporulation of the EPF was determined. In standard dip bioassay, the lowest LT_{50} of 10.4 days was induced by *L. lecanii*, while the wheat diet incorporation method resulted in LT_{50} values ranging between 13.1 and 15.2 days. The LC_{50} values for *E. globulus*, *F. vulgare*, and *T. ammi* were 162.3, 140.3, and 310 µl L^{-1} air against adults, respectively. The EOs examined showed strong inhibition of mycelial growth, conidial germination, and sporulation at sublethal concentrations. EOs of *F. vulgare* and *T. ammi* completely inhibited mycelial growth and sporulation of the tested EPF. Germination inhibition ranged from 100% in *L. lecanii* exposed to EO from *F. vulgare* to 52.3% in *B. bassiana* Z1 exposed to EO from *T. ammi*. Based on the results, although EOs and EPF are successful agents to control adults *T. castaneum* when used separately, it cannot be applied in combination because of the conflicting effect.

Keywords *Beauveria bassiana* · *Purpureocillium lilacinum* · *Lecanicillium lecanii* · Microbial control · Fumigant toxicity

Introduction

The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), has a world-wide distribution and is among the most economically important pest species in stored products. *T. castaneum* has a broad range of food preferences and is particularly abundant in flour mills, grocery shops, and stored grains (Garcìa et al. 2005; Lu et al. 2010). Insecticides are important components of insect pest management programs for stored grains, mills, processing plants, and retail stores (Arthur and Subramanyam 2012). However, the frequent and continuous application of synthetic chemical insecticides has resulted in serious drawbacks on the environment, toxicity hazards on non-target organisms, and the development of resistance (Isman 2006; Daglish 2008; Watts and Williamson 2015). These concerns along with consumer demand for less toxic pest insect control products have pushed research toward more ecologically compatible bio-agents for insect pest management.

One possible approach for safer stored products pest insect control is the use of essential oils (EOs) that show no or minimal off target effects (Rajendran and Sriranjini 2008; Cosimi et al. 2009). Essential oils are generally complex mixtures of organic compounds rich in monoterpenes which cause insect death by suppressing acetylcholinesterase activity (Houghton et al. 2006; Bakkali et al. 2008). These botanicals may have different types of action such as fumigant activity (Ilboudo et al. 2010; Nennah and Ibrahim 2011), contact toxicity (Taghizadeh-Saroukolai 2010; Kim et al. 2011), repellency (Celar and Kos 2016; Caballero-Gallardo et al. 2012), and anti-feedant activity (Stefanazzi et al. 2011). They may also induce changes in biological parameters such

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as growth rate, reproduction, and lifespan (Papachristos and Stamopoulos 2002).

Insecticidal efficacy has been shown with some EOs against adult *T. castaneum*. Islam et al. (2009) reported that fumigation of coriander (*Coriandrum sativum* L.) with EO (0.08 µg ml^{-1}) yields 100% mortality against larvae, pupae, and adults of *T. castaneum* at 96 h post-exposure, and 100% egg mortality with fumigation at a significantly higher level (20 µg ml^{-1}). The fumigant and contact toxicities of EOs from 20 different Egyptian plants against adults of *T. castaneum* and their inhibitory effects on acetylcholinesterase and adenosine triphosphatases have also been studied by Abou-Taleb et al. (2016).

Cosmopolitan entomopathogenic fungi (EPF) with their diverse range of insecticidal activity are used for integrated pest management in agriculture (Duarte et al. 2016). The application of these pathogenic agents is one of the most promising alternatives to traditional synthetic chemical insecticides as they combine high efficacy, low mammalian toxicity, and natural biological origins (Moore et al. 2000). EPF such as *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae), *Lecanicillium* (= *Verticillium*) *lecanii* (Zimm.) Zare & Gams (Hypocreales: Clavicipitaceae), and *Purpureocillium lilacinum* (Thom.) Luangsaard, Hou-braken, Hywel-Jones & Samson (Hypocreales: Ophiocordycipitaceae) are considered the most important EPF that have been used against a wide spectrum of insect pests (Ambethger 2009). These fungi have been shown to be pathogenic against adult *T. castaneum* in numerous laboratory trials. Golshan et al. (2014) tested nine isolates of *B. bassiana* against adult *T. castaneum* and showed that virulence is highly variable. Specifically, they showed that *B. bassiana* isolate IRAN 440C is the most virulent against *T. castaneum*, whereas isolate DEBI 014 showed the lowest median lethal time (LT_{50}). In another study, Shafighi et al. (2014) tested *B. bassiana* and *M. anisopliae* alone or in combination with diatomaceous earth (DE) against *T. castaneum*, *Rhyzopertha dominica* (F.), and *Oryzaephilus surinamensis* (L.) They found that DE enhances the insecticidal efficacy of these EPF.

By combining plant EOs and EPF, enhanced insecticidal efficacy may be accomplished thereby minimizing reliance on synthetic pesticide control and decreasing the risks of environmental contamination. However, the use of incompatible botanical insecticides may inhibit the germination and vegetative growth of the fungal biocontrol agent and adversely affect the overall IPM program (Hirose et al. 2001). The main goal of the present study was, therefore, to evaluate the compatibility of EOs isolated from six reputed medicinal plants (*Trachyspermum ammi* (L.) Sprauge ex Turrill (Apiacae), *Foeniculum vulgare* Mill. (Apiaceae), *Eucalyptus globulus* Labill. (Myrtaceae), *Salvia mirzayanii* Rech. F. & Esfand (Lamiaceae), *Majorana hortensis* Moench. (Lamiaceae), and *Thymus vulgaris* L. (Lamiacae)) with the EPF *L. lecanii*, *B. bassiana*, and *P. lilacinum*. The effects of these EOs alone or in combination with EPF on the mortality of *T. castaneum* were determined. The findings of this study will allow more effective application of these insecticidal compounds in IPM programs for *T. castaneum*.

Materials and methods

Plant material and extraction of essential oils

Plant parts including leaves and twigs of *S. mirzayanii*, *M. hortensis*, *T. vulgaris*, and *E. globulus* as well as the fruit of *F. vulgare* and *T. ammi* were collected from research fields of the Faculty of Agriculture (29° 22′ N, 51° 10′ E), Persian Gulf University, Bushehr Province, Bushehr, Iran. Plant parts of *S. mirzayanii*, *M. hortensis*, *T. vulgaris*, *F. vulgare* and *T. ammi* were collected during April and March, and those of *E. globulus* were collected during August 2019. The collected species were identified by comparison with existing herbarium specimens at Persian Gulf University. Plant materials were washed with distilled water and then air dried in the shade at 27 ± 1 °C. Plant parts were subjected to hydro-distillation for 3 h using a Clevenger-type apparatus (Goldis Company, Iran). The obtained EOs were dried over anhydrous sodium sulfate and stored at 4 °C prior to use in bioassays (Sohrabi and Kohanmoo 2017).

Fungi

Four fungal species were used in this study. *B. bassiana* isolate Z1, *P. lilacinum* isolate Iran 1026, and *L. lecanii* isolate Iran 229 were extracted from *Spodoptera exigua* Hubner (Lepidoptera: Noctuidae) in Nazlu, Urmia, Iran by Dr. Youbert Ghosta (University of Urmia, Iran). *B. bassiana* isolate IRAN1395C, was obtained from the Institute of Iranian Plant Protection in Tehran, Iran. These fungal strains were chosen for this study on the basis of their laboratory efficacy against other stored product pests like the waxworm (*Galleria mellonella* L.) and date sap beetle (*Carpophilus hemipterus* L.) that are commonly found in Iran (Sohrabi et al. 2019; Jamali et al. 2017). The fungi were cultured on potato dextrose agar (PDA) for the mass production of the conidia. During conidial production, culture plates were incubated at 25 ± 1 °C and 16 h illumination per day. Conidia were harvested by surface scraping 14-day-old sporulating cultures using a sterile scalpel and placed in a glass bottle containing 0.02% polyoxyethylene sorbitan monolaurate (Tween 80™; Merck). Spore suspensions were stirred vigorously on a shaker at 10,000 rpm for 5 min before being filtered through one layer of sterile jaconet. The concentration of fungal conidia in the homogenous conidial suspension was determined using a Neubauer haemocytometer (Precicolor, HBG; Germany). The conidial viability of fungal isolates was determined after 24 h as described by Lane et al. (1988). For all bioassays, the average viability of the conidia was over 95%.

Insect rearing

Red flour beetle adults were obtained from a laboratory colony and reared on wheat flour and yeast (10:1; w/w), at 28 ± 1 °C in darkness. Mixed-age adults were used in the bioassays.

Pathogenicity of fungi on adults of *T. castaneum*

Adult *T. castaneum* were exposed to the fungi using one of two application methods (i) conidial suspension (i.e., standard insect dip method) and (ii) wheat inoculated with conidial suspension (i.e., wheat diet incorporation method). The initial bioassay was performed using the standard insect dip method (Anonymus 1990). In brief, fifteen adults of *T. castaneum* were dipped into spore suspensions of *P. lilacinum*, *L. lecanii, B. bassiana* Z1, or *B. bassiana* IRAN1395C (concentrations of 1.52, 2.94, 2.98, and 3.07×10^9 conidia ml^{-1} of water containing 0.02% (v/v) Tween 80, respectively) for l0 s. Control insects were submerged in sterile distilled water containing 0.02% (v/v) Tween 80. Following exposure to the conidia, the treated insects were placed into cylindrical plastic containers (40 mm in diameter and 52 mm in height) with one screened hole (10 mm diameter) on the top of the container for ventilation. The container contained 10 g of sterilized partially damaged wheat as a food source.

The second bioassay was performed based on Kavallieratos et al. (2006) with some modifications. In this method, 50 g of sterilized damaged wheat was sprayed with 2 ml of the above mentioned conidia concentrations using a hand sprayer of 2000 ml capacity. Spraying was performed in a tray, on which the appropriate amount of wheat grain was spread into a thin layer. The treated grain was left to dry for 24 h post-spraying at room temperature (25 ± 1 °C). After the conidia-treated grain was completely dry, 10-g aliquots were placed in the cylindrical containers. Three additional containers, containing wheat treated with distilled water containing 0.02% (v/v) Tween 80, were used as a control. Fifteen *T. castaneum* adults were transferred into each container. All of the containers were incubated at 26 ± 1 °C, 70 ± 5% RH and a photoperiod of 14:10 (L:D). Three replicates were used for each fungus, and each experiment was repeated three times. The number of dead and live larvae were counted for 14 d every other day. Dead adults in all treatments were removed and surface sterilized with 2.5% sodium hypochlorite for 3 min, washed twice with sterile distilled water, and then incubated into Petri dishes on moistened filter paper for 3–5 days. Adults with fungal sporulation were considered to have died from the fungal infection.

Fumigant toxicity of essential oils against *T. castaneum*

In order to examine the fumigant toxicity of the EOs, 15 adults of *T. castaneum* were released into a closed cylindrical container (40 mm diameter by 52 mm height) containing one of six EO concentrations (133.33 to 800 µl L^{-1}) with three replicates for each concentration. In order to accomplish this, the desired concentration of each EO was applied on a 10-mm-diameter piece of Whatman No. 1 filter paper that was attached to the inner surface of the lid of the container. A filter paper disk without EO was placed in each control cylinder. The containers were sealed with parafilm, and mortality was determined after 24, 48, and 72 h from the commencement of the exposure. The bioassays were carried out at 26 ± 1 °C, 70 ± 5% RH, and a photoperiod of 14:10 (L: D).

Effect of essential oils on fungal mycelial growth, sporulation, and conidial germination of fungi

The antifungal properties of the most effective EOs against *T. castaneum* (determined from previous bioassay) were evaluated in terms of their volatile effects toward mycelial growth and sporulation as described by Soylu et al. (2007) and Nana et al. (2016) with some modifications. In brief, a conidial suspension (100 µl) containing 1×10^7 conidia ml^{-1} was spread on PDA plates (reference), and the plates were incubated at 25 ± 1 °C for 3 days in order to generate mycelial mats. The unsporulated mycelial mats were then cut into round agar plugs using a 5-mm-diameter cork borer. Subsequently, each agar plug was singly transferred onto the center of a fresh PDA agar plate. Petri plates (90 × 20 mm; Isolab, Iran) which provided 80 ml of air space after the addition of 20 ml agar medium were used for the determination of the volatile phase effect of EOs from *E. globulus*, *T. ammi*, and *F. vulgare*. An LC_{25} concentration (determined from previous experiments) was applied to a 10-mm-diameter disk of Whatman No. 1 filter paper and then placed on the inner surface of the inverted lid of the Petri dish. A filter paper disk that was not treated with EO was used as a control. Three Petri plates representing three replicates per treatment were used. The Petri dishes were sealed with parafilm and incubated in complete darkness at 25 ± 1 °C for 7 days. Radial growth of each fungus was recorded at seven days' post-treatment. The experiment consisted of three replicates, and each experiment was repeated twice on different days.

To assess conidial production, the sporulated mycelial mats were cut from the culture plates into agar plugs using a 5-mm-diameter cork borer. Each agar plug was then transferred singly into a bottle containing 10 ml of sterile distilled water containing 0.02% sterile Tween 80. The bottle was then vortexed for 4 min, and the spore concentration was determined using a Neubauer haemocytometer. The experiment consisted of four replicates and was repeated two times on different days.

To study the effect of EOs on the conidial germination of fungi, 100 µl of conidial suspension (1×10^7 conidia ml^{-1}) was spread on a water agar (0.9%) plate. An LC_{25} concentration of each EO was then added to a 10-mm-diameter disk of filter paper. Control plates were not treated with EOs. The plates were sealed with parafilm and incubated at 25 ± 1 °C in darkness. The percentage of germinated conidia was quantified at 24 h post-exposure to each EO. One hundred conidia were counted on a random basis for each Petri dish. Conidia were considered as germinated when the germ tube was longer than the conidial diameter (Marcuzzo and Eli 2016). Each treatment was replicated three times and repeated twice on different days.

Data analysis

Mortality data on the toxicity of fungi and EOs tested against *T. castaneum* adults were subjected to arcsin square root transformation before analysis. A $4 \times 2 \times 7$ factorial analysis of variance (ANOVA) (SAS Institute 2003) was applied to study the possible effects of the two application methods on the percentage mortality data of *T. castaneum* adults exposed to EPF isolates at different times. The analysis of variance model included the main effects of each fungus, method of application, lethal time, and the interaction of the main effects. The time necessary to produce 50% mortality (LT_{50}) was estimated by probit analysis (SAS Institute 2003). A $6 \times 6 \times 3$ factorial ANOVA (SAS Institute 2003) was also applied to study the possible effects of the EOs on the percentage mortality data of *T. castaneum* adults exposed to different concentrations at various times. This analysis of variance model included the main effects of the EO, EO concentration, lethal time, and the interaction of the main effects. Lethal concentration values (LC_{25}, LC_{50} and LC_{90}) and their corresponding 95% fiducial limits (FL) for each essential oil were also estimated by probit analysis (SAS Institute 2003). The fungus–essential oil compatibility data were analyzed according to the classification scheme of Ambethgar et al. (2009). The replicated fungal radial growth, sporulation, and spore germination data were averaged and expressed as percentage of growth, percentage sporulation, and percentage conidial germination inhibition in comparison with the corresponding control. The percentage of inhibition (*I*) of mycelial growth/sporulation/conidial germination inhibition was determined using the formula of Hokkanen and Kotiluoto (1992):

$$I(\%) = \frac{C - P}{C} \times 100$$

where *C* and *P* are mycelial growth/sporulation/conidial germination of fungus in the control medium and medium with EO, respectively. Four inhibition levels were used to evaluate the effect of the EO on EPF (Ambethger et al. 2009): 1 = harmless (< 25%), 2 = slightly harmful (25–35%), 3 = moderately harmful (36—50%) and 4 = harmful (> 50%). This classification takes into account that inhibition higher than 50% is scarcely justifiable since biological control agents are generally not as effective as chemical pesticides (Celar and Kos 2016). An arcsine square root transformation was performed on the percentage of mycelial growth/sporulation/conidial germination inhibition data before analysis. A 2×2 factorial analysis of variance (ANOVA) (SAS Institute, 2003) was applied to study the possible effects of the EOs on the mycelial growth/sporulation/germination inhibition percentage of fungi exposed to EOs. This analysis of variance model included primary effects of the fungus, EO, and the interaction of the primary effects. Means were separated by the Duncan's Multiple Range Test (DMRT) (P = 0.05).

Results

Virulence of EPF on adult *T. castaneum*

Orthogonal contrasts revealed that mortality was significantly different among different times ($F_{6, 466} = 4.26$; $P < 0.0003$). The interactive effect between lethal time and application method used was also significant ($F_{6, 466} = 5.14$; $P < 0.001$).

In the first experiment using the standard dip method, the lowest LT_{50} value of 10.4 days was found with *L. lecanii* (Table 1), while in the wheat diet incorporation method the median lethal times ranged between 13 and 15 days, and no significant differences were found in lethal times between the EPF (Table 2). No significant difference in cumulative mortality was observed between treatments (Tables 1 and 2).

Fumigant toxicity bioassay

Orthogonal contrasts indicated that mortality was significantly different among the EOs ($F_{5, 266} = 315.44$; $P < 0.001$), concentrations applied ($F_{5, 266} = 62.38$; $P < 0.001$), and among different times ($F_{2, 266} = 95.02$; $P < 0.001$). The difference between the EOs and concentration applied for each EO was significantly different ($F_{25, 266} = 15.51$; $P < 0.001$). The interactive effect between each EO and lethal time was also significant ($F_{10, 266} = 15.26$; $P < 0.001$).

The LC_{25}, LC_{50}, and LC_{90} values of the EOs tested are summarized in Table 3. These values indicated that *E. globulus* and

Table 1 Cumulative mortality at 14 d post-exposure and median lethal time (LT_{50}) of adult *Tribolium castaneum* following exposure to entomopathogenic fungal isolates using the standard dip method

Fungal isolate	% Mortality[a]	LT_{50} (d) (95% FL)[b]	Slope ± SE
Beauveria bassiana IRAN1395C	50.4 ± 5.8	13.91 (12.07–17.71)	3.21 ± 0.28
Beauveria bassiana Z1	51.9 ± 8.1	13.73 (12.42–15.77)	2.74 ± 0.29
Purpureocillium lilacinum	48.9 ± 6.6	14.59 (13.28–16.87)	3.80 ± 0.48
Lecanicillium lecanii	63.0 ± 7.8	10.38 (9.65–11.31)	2.65 ± 0.22

[a]Means ± SE ($n = 36$)

[b]Median lethal time and 95% fiducial limits (FL) were estimated using logistic regression (SAS Institute 2003) ($P < 0.001$ for all treatments)

Table 2 Cumulative mortality at 14 d post-exposure and median lethal time (LT_{50}) of adult *Tribolium castaneum* following exposure to entomopathogenic fungal isolates using the wheat diet incorporation method

Fungal isolate	% Mortality[a]	LT_{50} (d) (95% FL)[b]	Slope ± SE
Beauveria bassiana IRAN1395C	64.8 ± 9.6	13.08 (11.63–16.46)	4.54 ± 0.87
Beauveria bassiana Z1	55.9 ± 10.6	14.80 (13.31–18.58)	4.75 ± 0.97
Purpureocillium lilacinum	66.3 ± 7.4	14.02 (12.68–16.4)	3.57 ± 0.49
Lecanicillium lecanii	55.9 ± 13.1	15.16 (13.51–18.49)	3.73 ± 0.57

[a]Means ± SE ($n = 36$)

[b]Median lethal time and 95% fiducial limits (FL) were estimated using logistic regression (SAS Institute 2003) ($P < 0.001$ for all treatments)

Table 3 Lethal concentrations (LC_{25}, LC_{50}, and LC_{90}) of essential oils applied to adult *Tribolium castaneum*

Essential oil	LC_{25} (μl L^{-1} air) (95% FL)[a]	LC_{50} (μl L^{-1} air) (95% FL)	LC_{90} (μl L^{-1} air) (95% FL)	χ^2 (df)	Slope ± SE[b]
Eucalyptus globulus[b]	111.33 (76.67–140.0)	162.27 (126.67–192.93)	329.87 (277.73–421.07)	7.06 (4)	4.16 ± 0.65
Foeniculum vulgare	86.13 (0.4–163.07)	140.27 (5.6–233.33)	354.53 (206.93–2117.2)	12.54 (4)	3.18 ± 0.95
Trachyspermum ammi	235.2 (192–269.9)	310.0 (270.4–346.13)	524.0 (464.27–620)	4.36 (4)	5.62 ± 0.74

[a]Median lethal concentration and 95% fiducial limits (FL) were estimated using logistic regression (SAS Institute, 2003) ($P < 0.001$ for all treatments) ($n = 54$)

[b]The species name is the plant from which the essential oil originates

F. vulgare were approximately two times more effective than *T. ammi* against adult *T. castaneum*. Differences between the toxicity of the EOs from *E. globulus* and *F. vulgare* were not significant (Table 3). The median lethal concentrations (LC_{50}) of EOs from *E. globulus*, *F. vulgare*, and *T. ammi* against adult *T. castaneum* at 72 h post-exposure were 162, 140, and 310 μl L^{-1} air, respectively (Table 3). The mortality of adult *T. castaneum* caused by EOs from *S. mirzayanii*, *M. hortensis*, and *T. vulgaris* at 72 h post-exposure was too low to compute LC_{50} values. Mortality rates of adults *T. castaneum* at the highest concentration tested (800 μl L^{-1}) of these EOs were 13.33, 3.33, and 10.00%, respectively, at 72 h post-exposure.

Effects of EOs on inhibition of conidial germination, mycelial growth, and sporulation of EPF

Based on the results of the fumigant toxicity assays, EOs from *E. globulus*, *T. ammi*, and *F. vulgare* were chosen for further analysis of their effects on the germination of EPF. The germination inhibition percentage of the EPF tested in this study was significantly affected by fungal isolates ($F_{3, 60} = 22.99$; $P < 0.001$). The inhibitory effect of the EOs on the germination percentage of *L. lecanii* (96.7 ± 1.4) and *B. bassiana* IRAN1395C (92.9 ± 1.52) was significantly higher than that of *P. lilacinum* and *B. bassiana* Z1 (Table 4).

The growth inhibition percentage of fungal isolates was significantly affected by the EOs ($F_{2, 60} = 84.62$; $P < 0.001$). Mycelial growth inhibition ranged from 100% with EOs of *F. vulgare* and *T. ammi* to 82.1% with that of *E. globulus* (Table 4). Similarly, the sporulation of fungal isolates was significantly affected by the EOs ($F_{2, 84} = 59.82$; $P < 0.001$). Sporulation inhibition ranged from 100% with EOs of *F. vulgare* and *T. ammi* to 79.2% with that of *E. globulus* (Table 4).

Essential oils tested significantly inhibited mycelial growth, sporulation, and conidial germination of EPF, with all of them placed in the highest inhibition class 4 (Table 5).

Table 4 Primary effect of fungal isolates and essential oils on percentages of germination inhibition (GI), mycelial growth inhibition (MGI), and sporulation inhibition (SI) of entomopathogenic fungi

Fungal growth inhibition	Main effects	Main effect levels	Mean ± SE
GI	Fungal isolates	*Lecanicillium lecanii*	96.7 ± 1.4[a]
		Beauveria bassiana IRAN1395C	92.9 ± 1.52[a]
		Purpureocillium lilacinum	78.0 ± 3.9[b]
		Beauveria bassiana Z1	64.8 ± 4.9[c]
MGI	Essential oil	*Foeniculum vulgare*	100 ± 0.0[a]
		Trachyspermum ammi	100 ± 0.0[a]
		Eucalyptus globulus	82.1 ± 2.1[b]
SI	Essential oil	*Foeniculum vulgare*	100 ± 0.0[a]
		Trachyspermum ammi	100 ± 0.0[a]
		Eucalyptus globulus	79.2 ± 3.28[b]

Means for each trait followed by different letters are significantly different (DMRT, $P = 0.05$) ($n = 72$, 72, and 96 for GI, MGI, and SI, respectively)

F. vulgare and *T. ammi* inhibited mycelial growth and sporulation entirely at concentrations equivalent to LC25 (100% inhibition), while *E. globulus* had a smaller effect on mycelial growth and sporulation ranging from 74.2–86.6% and 71.9–88% inhibition, respectively.

Essential oils used had more inhibitory effects on conidial germination of *B. bassiana* IRAN 1395C and *L. lecanii* (ranging from 91.3–100% inhibition) in comparison with *B. bassiana* Z1 and *P. lilacinum* (ranging from 52.3–81.7% inhibition).

Discussion

The findings of the two bioassay methods used in the current study show that the four EPF (*B. bassiana* isolates Z1 and IRAN1395C, *L. lecanii* Iran 229, and *P. lilacinum* Iran 1026) tested in this study have strong potential to control adults of *T. castaneum*. These entomopathogenic fungi can be considered as promising agents for use in biocontrol programs. The pathogenicity of EPF has been previously documented against *T. castaneum* and other stored product pests

Table 5 Effects of essential oils at concentrations equivalent to LC_{25} on percentage of mycelial growth inhibition (MGI), germination inhibition (GI), and sporulation inhibition (SI) of entomopathogenic fungi

Fungal isolates	Essential oils[a]	Fungal growth factors					
		MGI[c]	Inhibition class[b]	GI[c]	Inhibition class	SI[c]	Inhibition class
Beauveria bassiana IRAN1395C	*E. globulus*	74.2 ± 6.0	4	94.36 ± 1.4	4	80.65 ± 6.9	4
	F. vulgare	100 ± 0.0	4	91.3 ± 3.8	4	100 ± 0.0	4
	T. ammi	100 ± 0.0	4	93.1 ± 2.5	4	100 ± 0.0	4
Beauveria bassiana Z1	*E. globulus*	80.7 ± 7.3	4	66.9 ± 7.4	4	71.86 ± 3.5	4
	F. vulgare	100 ± 0.0	4	75.3 ± 7.6	4	100 ± 0.0	4
	T. ammi	100 ± 0.0	4	52.3 ± 9.0	4	100 ± 0.0	4
Lecanicillium lecanii	*E. globulus*	86.8 ± 5.4	4	94.0 ± 3.6	4	88.0 ± 7.8	4
	F. vulgare	100 ± 0.0	4	100 ± 0.0	4	100 ± 0.0	4
	T. ammi	100 ± 0.0	4	96.2 ± 1.7	4	100 ± 0.0	4
Purpureocillium lilacinum	*E. globulus*	86.6 ± 2.3	4	73.0 ± 7.9	4	76.2 ± 6.9	4
	F. vulgare	100 ± 0.0	4	81.7 ± 3.7	4	100 ± 0.0	4
	T. ammi	100 ± 0.0	4	79.4 ± 8.3	4	100 ± 0.0	4

[a]The species name is the plant from which the essential oil originates

[b]Inhibition classes according to Ambethgar (2009): 1 = harmless (< 25%), 2 = slightly harmful (25–35%), 3 = moderately harmful (36–50%) and 4 = harmful (> 50%)

[c]Means ± SE ($n = 72$, 72, and 96 for MGI, GI, and SI, respectively)

(Michalaki et al. 2007; Wakil et al. 2014; Storm et al. 2016; Ashraf et al. 2017; Dal Bello et al. 2018). The pathogenicity of *L. lecanii* and *P. lilacinum* against adults of *T. castaneum* has been evaluated for the first time in the present study, although susceptibility of other stored product pests including *Sitophilus zeamais* Motsch. (Coleoptera: Curculionidae) and *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) to these fungi has been previously documented (Ahmed 2010; Barra et al. 2013). The efficacy of the *L. lecanii* IRAN 229 and *B. bassiana* Z1 isolates used in this study has recently been determined against several instar larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae) by Sohrabi et al. (2019). In the current study, we found that the LT_{50} value of *P. lilacinum* against *T. castaneum* was about 14 days with both bioassay methods that were tested. In another study, among 20 isolates of *P. lilacinum* that were tested, the shortest LT_{50} value was found to be 4.66 days when determined using three stored product pests including *T. confusum* (Barra et al. 2013). The differences in the LT_{50} values in our study and that of the other studies are likely due to different fungal isolates that were tested and/or differences in the virulence of the same isolates against various insect species.

In the present study, the toxicity of EOs obtained from six plant species was evaluated against *T. castaneum*; of these, EOs of *E. globulus*, *F. vulgare*, and *T. ammi* exhibited insecticidal activities. Insecticidal effects have been previously reported with EOs from various *Eucalyptus* spp. against major stored-grain insects including *T. castaneum*, *Callosobruchus maculatus* Fabr. (Coleoptera: Chrysomelidae), *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae), and *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) (Lee et al. 2004; Negahban and Moharramipour 2007; Nattudurai et al. 2012; Siddique et al. 2017). The repellency and toxicity of EOs from *T. ammi* and *F. vulgare* against larvae and adults of *T. castaneum* have also been reported previously by other researchers (Chaubey 2007a, b; Khorrami et al. 2018). The insecticidal activity of constituents of EO of *F. vulgare* including *(E)*-anethole, estragole, and fenchone against some stored product pests is largely attributable to fumigant activity rather than contact activity as reported by Kim and Ahn (2001). Our results showing insecticidal effects of EOs from *E. globules*, *F. vulgare*, and *T. ammi* on *T. castaneum* are consistent with those reported by other researchers. The insecticidal components of many plant essential oils are mainly monoterpenoids and their toxicity on different pests has been reported in previous studies (Regnault-Roger and Hamraoui 1995; Ibrahim et al. 2001; Kim and Ahn 2001). In the current study, the efficacy of *E. globulus* essential oil against *T. castaneum* might be attributed to its major component 1, 8-cineole (78.7%) (Sohrabi et al. 2015), which exhibits insecticidal activities against several insects (Liska et al. 2011; Pant et al. 2014). The main active compounds found in the EOs of *F. vulgare* and *T. ammi* are E anethole (76.8%) (unpublished data) and thymol (38.97%) (Sohrabi and Kohanmoo 2017), respectively. The repellency and insecticidal properties of thymol and E anethole have been proved in previous studies (Kim and Ahn 2001; Pandey et al. 2009; Bedini et al. 2016).

In the current study, EOs from *M. hortensis*, *S. mirzayanii*, and *T. vulgaris* showed unsatisfactory toxicity against adults of *T. castaneum*. There was insufficient mortality even to compute LC_{25} values. Similarly, Mohamed et al. (2008, 2009) stated that the oil of *M. hortensis* displayed very strong toxic activity by contact assay, while it showed no toxic effects by fumigant assay.

The fumigant toxicity of *T. vulgaris* against adult *T. castaneum* was also investigated in this study, and the mortality rate was too low. In previous researches, *T. castaneum* adults showed no susceptibility to the EOs from other *Thymus* species (Karabörklü et al. 2010; Taghizadeh-Saroukolai et al. 2010). In the present study, the fumigant toxicity of EO of *S. mirzayanii* was evaluated for the first time against adults of *T. castaneum*, and the oil exhibited little mortal effects. Same results have also been previously observed against *T. confusum* even under the highest concentration applied by Nikooei and Moharramipour (2010).

Our current results indicated that EOs from *E. globules*, *F. vulgare*, and *T. ammi* exhibit fumigant toxicity against EPF. Sublethal concentrations of the EOs significantly inhibited all the growth parameters tested including spore germination, radial growth, and conidial yield of isolates of *P. lilacinum*, *B. bassiana*, and *L. lecanii*. The use of incompatible EOs may inhibit the development and reproduction of EPF resulting in negative effects on integrated pest management strategies. Since germination is the first step in the infection process, compatibility between plant EOs and fungal spore germination should be considered as the most important factor when considering the use of these compounds (Anderson and Roberts 1983). Thus, if germination inhibition occurs, the fungal control efficiency will be affected by the EOs (Hirose et al. 2001).

The negative impact of EO of *E. globulus* and other *Eucalyptus* species on EPF including *B. bassiana* has been previously reported by other researchers (Immediato et al. 2016; Nardoni et al. 2018). In the current study, the finding that *E. globulus* was toxic to *B. bassiana* might be attributed to its major component 1, 8-cineole (Sohrabi et al. 2015), an oxygenated monoterpene which exhibits lower antifungal properties than phenolic compounds (Safaei-Ghomi and Ahd 2010; Nardoni et al. 2018).

In vitro antifungal activities of EOs of *T. ammi* and *F. vulgare* have been reported against several non-pathogenic fungal species (Abou-Jawdah et al. 2002; Mimica-Dukić et al. 2003; Singh et al. 2004; Soylu et al. 2005, 2006, 2007; Moein et al. 2014). To the best of our knowledge, this study is the first to show susceptibility of EPF to EOs of *T. ammi* and *F. vulgare*. The antimicrobial properties of EOs of *T. ammi* and *F. vulgare* and their major constituents thymol and anethole, respectively, have been shown to be able to suppress several human and plant pathogenic fungi (Mimica-Dukić et al. 2003; Soylu et al. 2006, 2007; Kordali et al. 2008; Moein et al. 2014).

Combining EPF and plant EOs as natural biocontrol agents may lead to fewer negative side effects compared to the use of synthetic chemical insecticides. However, according to our results, the volatile phases of the EOs used in this study negatively affected all growth factors of *B. bassiana*, *L. lecanii*, and *P. lilacinum* even at very low concentrations. The volatile phase of EOs has been reported to possess higher antimicrobial activity against plant pathogenic fungi and bacteria (Edris and Farrag 2003; Soylu et al. 2006, 2007). This higher antimicrobial activity likely originates from the ability of the fungal mycelium to easily absorb the naturally lipophilic EOs that are found in the vapor phase (Inouye et al. 2000; Edris and Farrag 2003).

Our findings suggest that the essential oils of *E. globulus*, *T. ammi*, and *F. vulgare* and entomopathogenic fungi *B. bassiana*, *L. lecanii*, and *P. lilacinum* can be used separately as valuable tools to control adults of *T. castaneum*. These agents, however, were not compatible when used in combination. However, future studies need to evaluate effects of these agents in the field applications, when they are either used in a sequence or rotation, or as applied to mixed populations as would occur in nature.

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