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Research Article/Araştırma Makalesi

Comparative evaluation of indigenous entomopathogenic fungal isolates and three commercial entomopathogenic fungal products against *Sitophilus oryzae* L. and *Tribolium confusum* du Val

Yerli entomopatojenik fungus izolatları ile üç ticari entomopatojenik fungus ürününün Sitophilus oryzae L. ve Tribolium confusum du Val üzerindeki etkinlikleri

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ABSTRACT

In the present study, a total of 17 indigenous entomopathogenic fungal (EPF) isolates (*Beauveria bassiana* (Bals.) Vuill. – 14, *Clonostachys rosea* (Link) Schroers – 2, *Isaria farinosa* (Holmsk.) Fr. – 1) obtained from soil samples collected from Antalya province (southwestern part of Turkey) and three commercial EPF products [i.e. Priority® (*Paecilomyces fumosoroseus*), Nibortem® (*Verticillium lecanii*) and Nostalgist® (*Beauveria bassiana*)] were evaluated for their efficacy against the 7–10-day-old adults of *Sitophilus oryzae* L. (Coleoptera: Curculionidae) and *Tribolium confusum* du Val. (Col.: Tenebrionidae) under laboratory conditions. All the isolates and products were tested at 1×10^7 conidia/ml suspensions against the both insect species. The results from the single-dose pathogenicity assays showed that three *B. bassiana* isolates (BbDm-1, BbKp-1 and BbMp-1) caused mortalities 96.7%, 100% and 93.3% in *S. oryzae*, and 100%, 100% and 96.7% in *T. confusum*, respectively, 14 days after inoculation whereas all three commercial products achieved mortalities ranging from 56.7% and 63.3% in *S. oryzae* and from 56.7% and 66.7% in *T. confusum*. In addition, the results from molecular phylogenetic analyses based on the ITS region sequence indicated that the three effective *B. bassiana* isolates (BbDm-1, BbKp-1 and BbMp-1) showed a high similarity (99%) with other *B. bassiana* isolates in GenBank. Overall results suggest that these three *B. bassiana* isolates have potential for management of *S. oryzae* and *T. confusum*.

Key Words: *Beauveria bassiana*, *Clonostachys rosea*, *Isaria farinosa*, *Sitophilus oryzae*, *Tribolium confusum*

ÖZ

Bu çalışmada, Antalya ilinden toplanan toprak örneklerinden izole edilen toplam 17 yerli entomopatojenik fungus (EPF) izolatı (*Beauveria bassiana* (Bals.) Vuill. - 14, *Clonostachys rosea* (Link) Schroers - 2, *Isaria* farinosa (Holmsk.) Fr. - 1 ve üç ticari EPF ürünü [ör. Priority® (*Paecilomyces fumosoroseus),* Nibortem® (*Verticillium lecanii*) ve Nostalgist® (*Beauveria bassiana*)] laboratuvar koşulları altında *Sitophilus oryzae* L. (Coleoptera: Curculionidae) ve *Tribolium confusum* du Val. (Col.: Tenebrionidae)'un 7-10 günlük erginlerine karşı etkinlikleri belirlenmiştir. Tüm EPF izolatları ve ticari ürünler, her iki böcek türüne karşı 1×10^7 conidia / ml süspansiyonlarda test edilmiştir. Tek doz patojenite testlerinin sonuçlarına göre uygulamadan 14 gün sonra sırasıyla, üç *B. bassiana* izolatı (BbDm-1, BbKp-1, BbMp-1) *S. oryzae*'de % 96.7, % 100, % 93.3 ve *T. confusum*'da % 100, % 100, % 96.7 ölüme neden olurken, ticari preparatlar ise *S. oryzae*'de % 56.7 ile % 63.3 arasında *ve T. confurusum*'da % 56.7 ile % 66.7 arasında değişen ölüm oranlarına neden olmuştur. Virulensliği yüksek olan üç *B. bassiana* izolatının (BbDm-1, BbKp-1 ve BbMp-1) ITS bölgesinin sekans dizilimleri belirlenmiş ve GenBank Blast programı kullanılarak yapılan filogenetik analizlerden elde edilen sonuçlar GenBank'taki diğer *B. bassiana* izolatları ile yüksek benzerlik (% 99) göstermiştir. Etki oranları yüksek olan entomopatojen fungus izolatlarının *S. oryzae* ve *T. confusum* ile mücadele de kullanılma potansiyellerinin olduğu düşünülmektedir.

Anahtar Kelimeler: *Beauveria bassiana*, *Clonostachys rosea*, *Isaria farinosa*, *Sitophilus oryzae*, *Tribolium confusum*

Introduction

Wheat and wheat flour are important sources of nutrients in many parts of the world as well as in Turkey. They are an important source of energy, carbohydrate, protein and fiber, as well as containing a range of micronutrients such as vitamin E, some of the B vitamins, magnesium, zinc, folic acid, antioxidants and phytochemicals (Veraverbeke and Delcour, 2002). People who eat whole grains as part of a healthy diet have a reduced risk of some chronic diseases (FAO, 2012; Gaesser, 2014). Humans cannot consume wheat in its raw state, so it undergoes a number of processing steps. Wheat and wheat flour are used for the production of popular foods, such as bulgur, bread, bakery products, couscous, pasta and snacks.

Wheat and wheat flour are stored by the manufacturers for short or long term protection under suitable conditions from production to consumption. However, there are many pests that cause significant losses in quality and quantity during this storage process; especially insects and mites cause significant damage in storages (Rajendran, 2002). These pests damage the products by gnawing, eating and breaking them and reduce their seed and commercial value. Annual loss ratio is approximately 10% in the world as well as in Turkey, accounting for about 100 million tons. Fifty percent of this damage is caused by insects. The loss rate can be up to 100% due to storage malfunctions or improper storage (Yıldırım et al., 2001).

In Turkey, the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), is one of the pests of primary infestation of stored wheat and very destructive. Confused and red flour beetles [*Tribolium confusum* Duv. and *T. castaneum* (Herbst) (Coleoptera: Tenebrionidae)] are major secondary pests of wheat flour and bakery products. Of these two *Tribolium* species, the first one is more common and dense species, due to having more tolerance to lower temperatures (Howe, 2008).

Control of these insects relies heavily on the use of gaseous synthetic insecticides and fumigants, which has led to some problems, such as ozone depletion, pest resurgence, pest resistance to insecticides and lethal effects on non-target organisms in addition to direct toxicity to users (Pimentel et al., 2008; Talukder, 2009; Boyer et al., 2012). Therefore, much effort has been focused on alternative control materials for potentially useful products as commercial insect-control agents. One of the leading alternative methods is biotechnological method such as mating disruption (Mamay et al. (2016), mass trapping (Mamay and Dağ, 2016) and attract & kill (Mamay and Mutlu, 2019a). Although many studies have addressed the potential toxicity of plant-based materials, especially essential oils and their components as protectants for stored products (Tunc et al., 2000; Erler, 2005; Campolo et al., 2018), the residue effect studies of some of them are still required. In addition, the sensory analysis of food treated with these materials should be evaluated since, although this aspect is a main concern for consumers, it has been often disregarded. Due to the negativities mentioned above, biological control methods have become a trend in recent years (Mamay and Mutlu, 2019b). Microbial agents have an important place among biological control agents including entomopathogens such as fungi, bacteria, viruses and nematodes (Alramadan and Mamay, 2019a, b, c, d). Entomopathogenic fungi (EPF) are common in terrestrial environments and play an important role in the regulation of insect populations. Therefore, they have been the subject of intensive research for more than 100 years (Lacey, 2017). In last three decades, they have been developed worldwide for the control of some insect pests, and today, some EPF products are already available commercially (Miller, 1995; Maina et al., 2018; Alramadan and Mamay, 2019a). However, there is increasing evidence that habitat selection drives the pathogenicity of EPF species (Bidochka et al., 2000). The objective of this study was to determine the efficacy of some indigenous isolates and commercial products of EPF against *S. oryzae* and *T. confusum* as potential biological control agents.

Material and Methods

Rearing of test insects

Sitophilus oryzae and *T. confusum* adults were obtained from their laboratory cultures maintained for about 5 years at the Plant Protection Department of Akdeniz University (Antalya, Turkey). While *S. oryzae* was reared on whole wheat kernels, *T. confusum* was reared on wheat flour including 5% brewer's yeast (by weight) at 26 ± 2°C and 65 ± 5% RH in continuous darkness. The 7–10-day-old adults of both species were used in the assays.

Indigenous isolates and commercial products of EPF

All indigenous EPF isolates used in the study were obtained from the EPF Collection of Plant Protection Department of Akdeniz University. They had previously isolated from soil samples collected from the selected agricultural habitats and their natural surroundings in Antalya province (southwestern part of Turkey) (Table 1). Although there is no commercial EPF agent specifically registered for *S. oryzae* or *T. confusum* (Batta and Kavallieratos, 2018), we chose three commonly used commercial EPF products as a comparison. The commercial EPF products, Priority® (*Paecilomyces fumosoroseus*, 1×10^9 cfu/ml), Nibortem® (*Verticillium lecanii*, 1×10^9 cfu/ml) and Nostalgist® (*Beauveria bassiana*, 1×10^9 cfu/ml) were purchased from the local companies in Antalya.

Table 1. Details of indigenous soil-borne entomopathogenic fungal isolates used in the study

Isolate code	Species	Origin	Vegetation	Latitude and longitude
BbKm-1	*Beauveria bassiana*	Kumluca	Olive	N 36°19'17.1" E 30°20'23.0"
BbKm-2	*B. bassiana*	Kumluca	Orange	N 36°22'18.8" E 30°16'29.1"
BbKr-1	*B. bassiana*	Kemer	Forest	N 36°35'51.0" E 30°33'22.7"
BbDm-1	*B. bassiana*	Demre	Orange	N 36°14'39.7" E 29°58'45.0"
BbFn-3	*B. bassiana*	Finike	Orange	N 36°19'53.7" E 30°08'40.6"
BbKp-1	*B. bassiana*	Kepez	Forest	N 36°54'50.4" E 30°37'48.4"
BbDs-2	*B. bassiana*	Döşemaltı	Pomegranate	N 37°00'02.4" E 30°38'16.1"
BbMp-1	*B. bassiana*	Muratpaşa	Fig	N 36°53'07.2" E 30°44'30.4"
BbAk-1	*B. bassiana*	Aksu	Grassland	N 36°56'03.3" E 30°52'35.1"
BbSr-1	*B. bassiana*	Serik	Orange	N 36°55'33.8" E 31°07'20.7"
BbMg-1	*B. bassiana*	Manavgat	Olive	N 36°49'40.8" E 31°20'35.3"
BbMg-2	*B. bassiana*	Manavgat	Wheat	N 36°58'58.8" E 31°14'48.5"
BbKl-1	*B. bassiana*	Korkuteli	Pear	N 37°03'21.3" E 30°10'33.8"
BbGp-1	*B. bassiana*	Gazipaşa	Forest	N 36°12'52.0" E 32°23'45.0"
CrMg-1	*Clonostachys rosea*	Manavgat	Wheat	N 36°57'49.2" E 31°16'51.9"
CrKn-1	*C. rosea*	Konyaaltı	Pear	N 36°53'52.7" E 30°37'50.8"
IfGp-1	*Isaria farinosa*	Gazipaşa	Olive	N 36°14'50.3" E 32°21'19.2"

The EPF isolates were cultured in Petri dishes (90 mm diameter) including Sabouraud Dextrose Agar (SDA, Merck, 108339) medium under laboratory conditions (25 ± 1°C, 75 ± 5% RH, and 12:12-h L/D) for 14 days. Conidia were collected from 10 to 14 day-old cultures by scraping inside surfaces of dishes with a sterile scalpel into 15 ml sterilized water containing 0.05% Tween-20 (Sigma-Aldrich®, St. Louis, MO). The conidial suspensions were mixed using a benchtop homogenizer (Vortex, Bohemia, New York) and a hemocytometer was used to determine the concentration of conidia. The spore concentration of each of the EPF isolates was adjusted to 1×10^7 conidia/ml before using in the assays. The same conidial concentration was prepared for each of the commercial EPF products. Distilled sterile water containing 0.05% Tween-20 was used as control.

Bioassays

Two parallel experiments were conducted according to method described by Kassaye (2010). For each of them, 30 adults of *S. oryzae* or *T.*

confusum in small nylon gauze bags were dipped in each treatment solution for 5 seconds, and then treated insects were placed in Petri dishes (disposable plastic 90 × 15 mm) lined with filter paper (Whatman® no: 1). Control insects were treated with sterile distilled water containing 0.05% Tween-20. All the dishes were sealed with Parafilm® M (Bemis, Neenah, WI) to prevent escape of insects and kept in an incubator at 27 ± 1°C and 70 ± 3% relative humidity for 24 h (Adane et al., 1996). After incubation, all insects were removed from the dishes and transferred to clean ones containing 20 g of food (whole wheat kernels for *S. oryzae* or wheat flour including 5% brewer's yeast for *T. confusum*). Then, all dishes were covered with Parafilm and returned to the incubator, as described above. The dishes (control and treated insects) were checked after 5, 7, 9 and 14 days and dead insects were counted and collected. At each observation, insects were touched using forceps and if the insect did not move, it was recorded as dead. To assess the growth of fungal mycelium on the insects, which would indicate insect mortality caused by the EPF agents, all dead insects were removed from the dishes and placed in new dishes lined with moistened filter paper, incubated at 26±2°C, and evaluated for up to 14 days under a stereomicroscope to observe fungal growth on the cadavers. In all experiments, each petri dish contained 10 unsexed adults of *S. oryzae* or *T. confusum* and was considered as one replicate. Three replicates were used for each treatment, and experiments were repeated two times with one-month interval. Thus, a total of 6 replicates were used for each treatment throughout the study.

Phylogenetic analysis

Considering the results from bioassays, genomic DNA of the most virulent EPF isolates (BbDm-1, BbKp-1 and BbMp-1) were extracted following the modified CTAB method described by Doyle and Doyle (1990). The PCR was performed in a Gradient Thermal Cycler by using two different primers based on ITS-rDNA region gene sequences which included, ITS1 (5'-TCCGTAGGTGAACCTGCGG- 3') and ITS4 (5' TCCTCCGCTTATTGATATGC-3') (White et al., 1990). The amplified PCR products were sequenced using the ABI 3730XL Sanger sequencing device (Applied Biosystems, Foster City, CA) and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) in the Macrogen Netherlands laboratory. The DNA sequences of these three *B. bassiana* isolates were performed using the ClustalW algorithm in the Bioedit program (Thompson et al., 1994; Hall, 1999). The nucleotide sequence of these isolates was compared with that of the other isolates of the related species using a Blast Bioinformatics search of sequences in the NCBI Genbank (Altschul et al., 1997).

Molecular phylogenetic analyses were conducted with MEGA5 software (Biodesign Institute, Tempe, Arizona) using the Maximum Likelihood method based on the Tamura 3-parameter model (Kimura, 1980; Tamura 1992). These analyses were done based on the ITS region sequence of the above-mentioned *B. bassiana* isolates and the nucleotide sequence of the other *B. bassiana* isolates retrieved from GenBank.

Analysis of mortality data

In all cases, no control mortality was observed and, therefore, no correction was necessary for the mortality data. All values were arcsine transformed prior to analysis. Data were analyzed by two-way ANOVA using the general linear model of the SPSS 23.0 Windows (IBM Corp. 2015, New York, USA). Differences among the treatment means were compared using the Tukey's multiple comparison test at a significance level of $P < 0.05$.

Results

Effectiveness of EPF isolates and products on Sitophilus oryzae

The results of the pathogenicity tests with the 7–10-day-old adults of *S. oryzae* showed that all tested EPF isolates and commercial products had

different efficacy rates against adult *S. oryzae* (Table 2). Mortality rates caused by isolates and products varied over time, and differences in mortality at each count date were generally significant among the different fungal isolates and products ($P < 0.05$). Of all the EPF isolates and products tested, three *B. bassiana* isolates (BbDm-1, BbKp-1 and BbMp-1) were most pathogenic and caused mortalities 96.7%, 100% and 93.3%, respectively, in adult *S. oryzae* at the longest incubation time (14 days after application). Even, at a shorter incubation time (9 days after application), these three isolates exhibited mortalities 80%, 73.3% and 80%, respectively. All the remaining ones had lower mortality rates than 65% even at the longest incubation time (Table 2).

Table 2. Mean mortality (%) of *Sitophilus oryzae* adults exposed to indigenous isolates and commercial products of entomopathogenic fungi 5, 7, 9 and 14 days after application

Fungal species	Isolate/Product name	Mean percent mortality (±SE) from a single dose trial*			
		5 days	7 days	9 days	14 days
Beauveria bassiana	BbKm-1	13.3±3.3^{abcB}	20.0±5.7^{cdB}	40.0±10.0^{bcAB}	60.0±10.0^{bcdA}
B. bassiana	BbKm-2	6.7±3.3^{abcC}	23.3±6.6^{bcdBC}	40.0±5.7^{bcAB}	60.0±5.7^{bcdA}
B. bassiana	BbKr-1	3.3±3.3^{bcB}	36.7±6.6^{abcA}	46.7±8.8^{abcA}	60.0±5.7^{bcdA}
B. bassiana	BbDm-1	20.0±0.0^{aC}	63.3±6.6^{aB}	80.0±5.7^{aAB}	96.7±3.3^{abA}
B. bassiana	BbFn-3	3.3±3.3^{bcB}	20.0±5.7^{cdAB}	40.0±5.7^{bcA}	46.7±8.81^{dA}
B. bassiana	BbKp-1	16.7±3.3^{abD}	50.0±5.7^{abC}	73.3±3.3^{abB}	100±0.0^{aA}
B. bassiana	BbDs-2	10.0±0.0^{abcC}	30.0±0.0^{bcBC}	46.7±3.3^{abcAB}	56.7±8.81^{cdA}
B. bassiana	BbMp-1	16.7±3.3^{abD}	63.3±3.3^{aC}	80.0±0.0^{aB}	93.3±3.33^{abcA}
B. bassiana	BbAk-1	0.0±0.0^{cB}	20.0±0.0^{cdAB}	30.0±10.0^{cdA}	46.7±6.7^{dA}
B. bassiana	BbSr-1	6.7±3.3^{abcC}	20.0±5.7^{cdBC}	36.7±3.3^{cAB}	43.3±6.7^{dA}
B. bassiana	BbMg-1	13.3±3.3^{abcB}	36.7±6.7^{abcA}	36.7±3.3^{cA}	50.0±5.7^{dA}
B. bassiana	BbMg-2	10.0±0.0^{abcB}	40.0±5.7^{abcA}	40.0±5.7^{bcA}	43.3±6.7^{dA}
B. bassiana	BbKl-1	3.3±3.3^{bcB}	26.7±3.3^{bcdAB}	40.0±11.5^{bcA}	46.7±6.7^{dA}
B. bassiana	BbGp-1	10.0±5.7^{abcB}	20.0±5.7^{cdAB}	43.3±8.8^{bcAB}	56.7±13.3^{cdA}
Clonostachys rosea	CrMg-1	6.7±3.3^{abcB}	36.7±6.7^{abcAB}	36.7±8.8^{cAB}	43.3±8.81^{dA}
C. rosea	CrKn-1	10.0±0.0^{abcC}	33.3±3.3^{bcB}	46.7±3.3^{abcAB}	56.7±6.6^{cdA}
Isaria farinosa	IfGp-1	3.3±3.3^{bcB}	26.7±6.7^{bcdAB}	33.3±8.8^{cdAB}	60.0±10^{bcdA}
Paecilomyces fumosoroseus	Priority®	13.3±3.3^{abcB}	36.7±6.7^{abcAB}	46.7±8.8^{abcA}	63.3±3.3^{abcdA}
Verticillium lecanii	Nibortem®	6.7±3.3^{abcC}	26.7±3.3^{bcdB}	40.0±0.0^{bcB}	60.0±5.7^{bcdA}
Beauveria bassiana	Nostalgist®	10.0±0.0^{abcC}	26.7±6.7^{bcdBC}	33.3±3.3^{cdB}	56.7±3.3^{cdA}
Control (dH water+Tween-20		0.0±0.0^{cA}	0.0±0.0^{dA}	0.0±0.0^{dA}	0.0±0.0^{eA}

*In single dose trial, all the isolates and products were tested at a concentration of 1×10^7 conidia/ml.
Means in a column followed by the same lower-case letter are significantly different ($P < 0.05$; Tukey test).
Means in a row followed by the same upper-case letter are significantly different ($P < 0.05$; Tukey test).

Effectiveness of EPF isolates and products on Tribolium confusum

Bioassays with the 7–10-day-old adults of *T. confusum* showed that both indigenous isolates and commercial products of EPF included in the study had variable pathogenicity against the pest and exhibited significant lethal effects compared to the control group ($P < 0.05$) (Table 3). The effectiveness was generally material (isolate/product) and time dependent. Of the seventeen EPF isolates tested, three *B. bassiana* isolates (BbDm-1, BbKp-1 and BbMp-1) were found the most virulence against adult *T. confusum* and caused 100%, 100% and 96.7% mortalities, respectively, 14 days after inoculation. These three isolates also had mortalities more than 70% at a shorter incubation time (9 days after application). Interestingly, none of the three commercial EPF products tested could cause mortalities ≥70% even at the longest incubation time (14 days after application).

Table 3. Mean mortality (%) of *Tribolium confusum* adults exposed to indigenous isolates and commercial products of entomopathogenic fungi 5, 7, 9 and 14 days after application

Fungal species	Isolate/Product name	Mean percent mortality (±SE) from a single dose trial*			
		5 days	7 days	9 days	14 days
Beauveria bassiana	BbKm-1	6.7±3.3^{cdeC}	13.3±3.3^{defBC}	36.7±.8.8^{bAB}	50.0±5.7^{cdA}
B. bassiana	BbKm-2	13.3±3.3^{cdeC}	20.0±5.7^{defBC}	36.7±3.3^{bB}	63.3±3.3^{cdA}
B. bassiana	BbKr-1	16.7±6.7^{bcdeB}	33.3±3.3^{bcdB}	40.0±5.7^{bAB}	60.0±5.7^{cdA}
B. bassiana	BbDm-1	36.7±3.3^{aC}	53.3±6.7^{abC}	76.7±3.3^{aB}	100±0.0^{aA}
B. bassiana	BbFn-3	6.7±3.3^{cdeC}	16.7±3.3^{defBC}	30.0±5.7^{bB}	56.7±6.7^{cdA}
B. bassiana	BbKp-1	23.3±3.3^{abcD}	43.3±3.3^{abcC}	76.7±3.3^{aB}	100±0.0^{aA}
B. bassiana	BbDs-2	13.3±3.3^{cdeC}	20.0±5.7^{defBC}	33.3±3.3^{bB}	56.7±3.3^{cdA}
B. bassiana	BbMp-1	33.3±3.3^{abD}	60.0±0.0^{aC}	73.3±3.3^{aB}	96.7±3.3^{abA}
B. bassiana	BbAk-1	3.3±3.3^{deC}	20.0±5.7^{defBC}	26.7±6.7^{bcAB}	46.7±3.3^{cdA}
B. bassiana	BbSr-1	6.7±3.3^{cdeB}	20.0±5.7^{defAB}	33.3±6.7^{bAB}	50.0±11.5^{cdA}
B. bassiana	BbMg-1	13.3±3.3^{cdeB}	23.3±6.7^{cdeAB}	33.3±6.7^{bAB}	53.3±12^{cdA}
B. bassiana	BbMg-2	6.7±3.3^{cdeC}	13.3±3.3^{defBC}	23.3±3.3^{bcAB}	33.3±3.3^{dA}
B. bassiana	BbKl-1	0.0±0.0^{eC}	6.7±3.3^{efBC}	16.7±3.3^{bcB}	43.3±3.3^{cdA}
B. bassiana	BbGp-1	6.7±3.3^{cdeB}	10.0±5.7^{efB}	23.3±6.7^{bcB}	60.0±10^{cdA}
Clonostachys rosea	CrMg-1	3.3±3.3^{deC}	13.3±3.3^{defBC}	20.0±0.0^{bcB}	56.7±3.3^{cdA}
C. rosea	CrKn-1	6.7±3.3^{cdeC}	16.7±3.3^{defC}	36.7±3.3^{bB}	50.0±0.0^{cdA}
Isaria farinosa	IfGp-1	3.3±3.3^{deB}	10.0±0.0^{efB}	20.0±5.7^{bcB}	50.0±5.7^{cdA}
Paecilomyces fumosoroseus	Priority®	16.7±3.3^{bcdeB}	23.3±3.3^{cdeB}	33.3±8.81^{bAB}	56.7±.8.8^{cdA}
Verticillium lecanii	Nibortem®	10.0±5.7^{cdeC}	16.7±3.3^{defC}	36.7±3.3^{bB}	66.7±3.3^{bcA}
Beauveria bassiana	Nostalgist®	20.0±0.0^{abcdC}	23.3±3.3^{cdeC}	43.3±3.3^{bB}	63.3±3.3^{cdA}
Control (dH water+Tween-20)		0.0±0.0^{eA}	0.0±0.0^{fA}	0.0±0.0^{cA}	0.0±0.0^{eA}

*In single dose trial, all the isolates and products were tested at a concentration of 1×10^7 conidia/ml.

Means in a column followed by the same letter are significantly different ($P < 0.05$; Tukey test).

Means in a row followed by the same letter are significantly different ($P < 0.05$; Tukey test).

Phylogenetic placement of the three most virulent EPF isolates

The DNA sequences of the three *B. bassiana* isolates (BbDm-1, BbKp-1 and BbMp-1) that had the highest virulence against both *S. oryzae* and *T. confusum* adults in pathogenicity tests were loaded into GenBank and the accession numbers were obtained and used for comparison in phylogenetic analysis. The accession numbers of the isolates are given in Table 4. After alignment, the ITS region sequence data set consisted of 487 aligned positions for *Beauveria* isolates. All the *B. bassiana* isolates from Turkey and GenBank were clustered together. The three *B. bassiana* isolates had high evolutionary homology with other *B. bassiana* isolates from the GenBank (Figure 1).

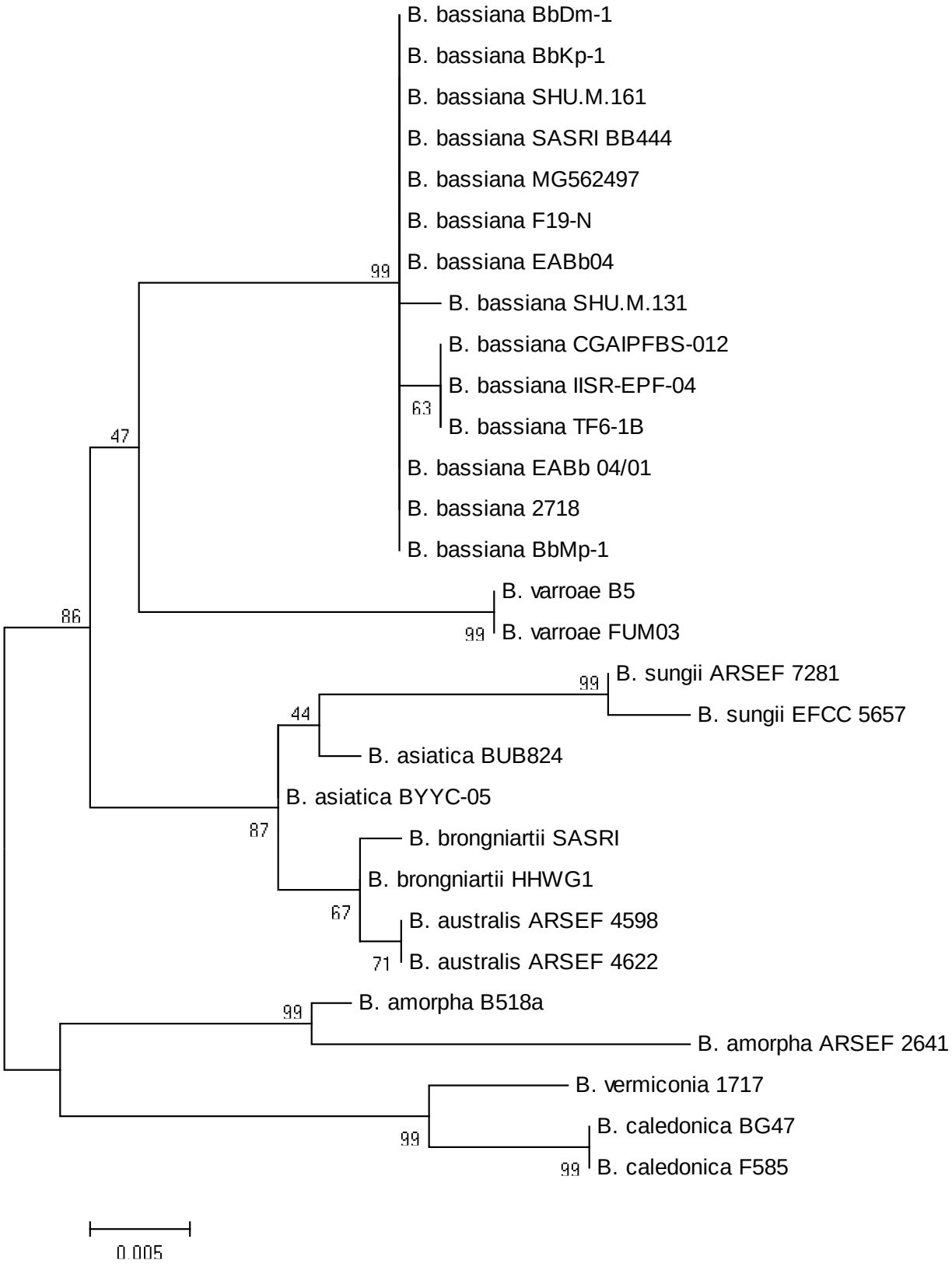

Figure 1. The Maximum Likelihood tree based on the Tamura 3-parameter model showing the phylogenetic relationship between the three *Beauveria bassiana* isolates (BbDm-1, BbKp-1 and BbMp-1) found to have high virulence in the present study and other *Beauveria bassiana* isolates from GenBank based on ITS region sequence

Table 4. GenBank nucleotide accessions of the three *Beauveria bassiana* isolates (BbDm-1, BbKp-1 and BbMp-1) that were the most virulent EPF isolates in pathogenicity bioassays together with other *Beauveria bassiana* isolates retrieved from the GenBank*

Isolate name	Species	Gene	Accession no
BbDm-1	*Beauveria bassiana*	ITS	MT441872
BbKp-1	*B. bassiana*	ITS	MT441877
BbMp-1	*B. bassiana*	ITS	MT441880
F19-N	*B. bassiana*	ITS	MG640376.1
MG562497	*B. bassiana*	ITS	MG562497.1
SHU.M.161	*B. bassiana*	ITS	KU158472.1
SHU.M.131	*B. bassiana*	ITS	KU158461.1
EABb04	*B. bassiana*	ITS	KC753382.1
SASRI BB444	*B. bassiana*	ITS	JX110368.1
TF6-1B	*B. bassiana*	ITS	JX122736.1
EABb 04/01	*B. bassiana*	ITS	DQ364698.1
CGAIPFBS-012	*B. bassiana*	ITS	KY495188.1
IISR-EPF-04	*B. bassiana*	ITS	KU363833.1
2718	*B. bassiana*	ITS	KU364353.1
HHWG1	*B. brongniartii*	ITS	JX110385.1
FUM03	*B. varroae*	ITS	MF667767.1
B5	*B. varroae*	ITS	MH374536.1
SASRI	*B. brongniartii*	ITS	JX110388.1
ARSEF 2641	*B. amorpha*	ITS	HQ880808.1
B518a	*B. amorpha*	ITS	HQ880806.1
BYYC-05	*B. asiatica*	ITS	MG345071.1
BUB824	*B. asiatica*	ITS	MG642836.1
ARSEF 4622	*B. australis*	ITS	HQ880790.1
ARSEF 4598	*B. australis*	ITS	HQ880789.1
F585	*B. caledonica*	ITS	DQ529233.1
BG47	*B. caledonica*	ITS	MT180427.1
1717	*B. vermiconia*	ITS	FJ973063.1
ARSEF 7281	*B. sungii*	ITS	HQ880815.1
EFCC 5657	*B. sungii*	ITS	JX463219.1

*ITS region sequence was used to determine the genetic diversity among the isolates

Discussion

Although some previous studies have demonstrated the occurrence of EPF on insect pests of stored-grains and their by-products (Odour et al., 2000; Mar et al., 2012; Barra et al., 2013; Er et al., 2016; Batta and Kavallieratos, 2018), as far as we know, no commercial bio-pesticides based on EPF bio-agents are registered for use against the stored-product insect pests. Also, there is no integration of any effective strain (formulated or unformulated) of EPF in the management of stored-product insects. The results from the present study indicate that screening of potential EPF isolates should not be limited to those isolated from the original host. Our findings suggest that indigenous soil-borne EPF isolates may suppress the populations of both species and may provide an alternative to gaseous synthetic insecticides and fumigants used in their control.

A review of the literature revealed that there are some studies indicating that EPF can be used as microbial control agents against the stored product insect pests in silo or other similar environments. For instance, Kavallieratos et al. (2014) tested indigenous soil-borne *B. bassiana* against <2 weeks old adults of *S. oryzae* at two different concentrations (2.11×10^7 and 2.11×10^8) in Greece. In the study, suspensions were applied by three treatments: (i) sprayed on adults of *S. oryzae* and set in petri dishes with food, (ii) sprayed on adults of *S. oryzae* and set in petri dishes without food, and (iii) sprayed on food and set in petri dishes with adults of *S. oryzae*. The mortality of *S. oryzae* adults during the overall exposure period for the lowest, as well as for the highest, concentrations of *B. bassiana* ranged from 0 to 100%. Both in the highest and the lowest concentrations of fungus, the mortality of *S. oryzae* adults was higher when the fungus was

applied on adults than when it was applied on food. Higher mortality was observed when food was absent than when food was present, in most of the cases tested. After 14 days of exposure, all adults were dead at both concentrations studied. Researchers reported that the high efficacy levels recorded in their study indicate that the tested *B. bassiana* isolate could be effective biocontrol agent against *S. oryzae*. In another study by Komaki et al. (2017), seven EPF isolates (*B. bassiana* (ARSEF-4984); *Paecilomyces farinosus* (ARSEF-2538); *Isaria fumosorosea* (ARSEF-4501); *I. farinosa* (ARSEF-3580); *Lecanicillium muscarium* (ARSEF-972 and ARSEF-5128), Mycotal extract of *L. muscarium* (as positive control) and distilled sterile water with Tween-20 (as negative control) were tested against *T. confusum* adults. All EPF isolates were sprayed at two different concentrations (1×10^5 and 1×10^7 conidia/ml) on adult insects in petri dishes. The results from the study demonstrated that the mortality rates of *T. confusum* adults treated with seven EPF isolates varied from 34.6 to 100% after 10 days of exposure. The highest mortalities of *T. confusum* adults were observed for *P. farinosus* (ARSEF-2538) with 100% mortality at 1×10^7 conidia/ml and *I. farinosa* (ARSEF-3580) with 97.3% mortality at 1×10^7 conidia/ml, followed by *I. fumosorosea* (ARSEF-4501), *B. bassiana* (ARSEF-4984) and *L. muscarium* (ARSEF-5128) with 94.6% mortality. Unlike their findings, we found that *I. farinosa* (IfGp-1) isolate obtained from soil samples collected from Antalya province and tested at 1×10^7 conidia/ml in this study had a low mortality rate (20% after 9 days of treatment) against *T. confusum* adults.

Compared to other insect pests, the application of EPF for the control of stored product insect pests is likely to have important limitations. However, some previous studies indicate that unformulated EPF can be applied on stored product insect pests by using aqueous conidial suspensions with different concentrations either by immersing the immature stages or adults of insects in these suspensions or by spraying the inner surfaces of grain containers before introduction of grains and insects (Moino et al., 1998; Sheeba et al., 2001; Padin et al., 2002; Lord, 2009; Khashaveh et al., 2011). The review of existing literature also revealed that EPF can be applied in combination with non-toxic natural products and the combinations of EPF and non-toxic natural products may serve as alternative control measures to synthetic insecticides against stored-grain insects.

Some previous studies indicated that certain combinations of EPF with natural products, such as chalk powder, oven ash, charcoal and diatomaceous earths yielded good results in the control of *S. oryzae* and *T. castaneum* by treating the inner surfaces of containers before introducing the grains and insects (Batta, 2004, 2008; Batta and Abu Safieh, 2005; Stephou et al., 2012). Although some researchers have reported that combinations of EPF and chemical insecticides can be used against the stored grain insects, very few of these combinations were effective, causing higher mortality to target insect species than the treatments with the EPF alone or the insecticide alone (Dal-Bello et al., 2000; Cherry et al., 2007). Considering all these reports, in the present study we applied both unformulated and formulated EPF by immersing adults of both *S. oryzae* and *T. confusum* in their prepared suspensions.

Finally, the present study revealed that some indigenous strains of soil-borne *B. bassiana* were more effective to adult *S. oryzae* and *T. confusum* than the foreign origin commercial EPF products tested. Our results also indicated that the use of EPF, in particular indigenous strains of *B. bassiana*, should be seriously considered for biological control because they have provided encouraging results for the control of both economic pests.

Conclusion

In conclusion, the present study showed that three *B. bassiana* isolates (BbDm-1, BbKp-1 and BbMp-1) recovered from soil samples and used in laboratory bioassays caused high mortality in

adult *S. oryzae* and *T. confusum* compared to the other tested fungal isolates and commercial products. For these reasons, the use of these three *B. bassiana* isolates might be a useful component in an integrated pest management (IPM) program against both insect species. Further research may be carried out to test the usefulness and effectiveness of these *B. bassiana* isolates in the field.

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