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

Yerli entomopatojenik funksiy izolatları ile üç ticari entomopatojenik funksiy 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* (Verticillum 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 × 10³ 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 funksiy (EPF) izolatı (*Beauveria bassiana* (Bals.) Vuill. – 14, *Clonostachys rosea* (Link) Schroers – 2, *Isaria farinosa* (Holmsk.) Fr. – 1) alınmıştır. Antalya ilinin (Batı Türkiye) toprak örneklerinden izole edilen toplam 17 yerli entomopatojenik funksiy (EPF) izolatı (BbDm-1, BbKp-1 ve BbMp-1) yarıştırıcılar üzerinde etkili olup, % 96.7, % 100 ve % 93.3 olarak belirtilmiştir. Atık ürünler, 14 gün sonra sırasıyla, % 100, % 100 ve % 96.7 olarak belirtilmiştir. Tüm EPF isolatları ve atık ürünler, her iki böcek türüne karşı 1 × 10³ konidio/ml süspansiyonlar test edilmiştir. Tek doz patojenite testlerinin sonuçlarına göre uygulamadan 14 gün sonra sırasıyla, üç *B. bassiana* isolatı (BbDm-1, BbKp-1 ve BbMp-1) % 96.7, % 100 ve % 96.7 olarak belirtilmiştir. Örneklerin genelde etkili olduğu düşünülmektedir.istrya 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* (Verticillum 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 × 10³ 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*.
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 × 10⁹ cfu/ml), Nibortem® (Verticillium lecanii, 1 × 10⁹ cfu/ml) and Nostalgist® (Beauveria bassiana, 1 × 10⁹ 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 30°16'29.1" |
| 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 × 10⁷ 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 x 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’-TCCGTAAGGTGAACTGCGG-3’) and ITS4 (5’ TCCTCCGCTTTATGATATGC-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**cD** | 40.0±10.0**DcAB** | 60.0±10.0**dcAB** |
|                        | BbKm-2               | 6.7±3.3**abcC** | 23.3±6.6**bcDBC** | 40.0±5.7**bcAB** | 60.0±5.7**bcAB** |
|                        | BbKr-1               | 3.3±3.3**abcB** | 36.7±6.6**bcDA** | 46.7±8.8**bcA** | 60.0±5.7**bcDA** |
| *B. bassiana*          | BbDm-1               | 20.0±0.0**cC** | 63.3±16.6**bA** | 80.0±5.7**bA** | 96.7±3.3**bA** |
|                        | BbFn-3               | 3.3±3.3**abcB** | 20.0±5.7**cD** | 40.0±5.7**bcA** | 46.7±8.8**bcA** |
| *B. bassiana*          | BbKp-1               | 16.7±3.3**abcB** | 50.0±5.7**bA** | 73.3±3.3**bA** | 100.0±0.0**a** |
|                        | BbDs-2               | 10.0±0.0**abcD** | 30.0±0.0**ABC** | 46.7±3.3**abcA** | 56.7±8.1**cDA** |
| *B. bassiana*          | BbMp-1               | 16.7±3.3**abcB** | 63.3±3.3**bcC** | 80.0±0.0**a** | 93.3±3.3**abcA** |
| *B. bassiana*          | BbAk-1               | 0.0±0.0**a** | 20.0±0.0**DdA** | 30.0±10.0**CD** | 46.7±6.7**CDA** |
| *B. bassiana*          | BbSr-1               | 6.7±3.3**abcC** | 20.0±5.7**bC** | 36.7±3.3**cA** | 43.3±6.7**CDA** |
| *B. bassiana*          | BbMg-1               | 13.3±3.3**abcB** | 36.7±6.7**abA** | 36.7±3.3**cA** | 50.0±15.7**CDA** |
| *B. bassiana*          | BbMg-2               | 10.0±0.0**abcB** | 40.0±5.7**bA** | 40.0±5.7**bA** | 43.3±6.7**CDA** |
| *B. bassiana*          | BbKl-1               | 3.3±3.3**abcB** | 26.7±3.3**bcD** | 40.0±11.5**SA** | 46.7±6.7**CDA** |
| *B. bassiana*          | BbGp-1               | 10.0±15.7**cB** | 20.0±5.7**cD** | 43.3±8.8**bcD** | 56.7±13.3**cDA** |
| *Clonostachys rosea*   | CrMg-1               | 6.7±3.3**abcB** | 36.7±6.7**abA** | 36.7±8.8**AB** | 43.3±8.1**CDA** |
| *C. rosea*             | CrKn-1               | 10.0±0.0**abcC** | 33.3±3.3**bcB** | 46.7±3.3**abcA** | 56.7±6.6**cDA** |
| *Isaria farinosa*      | IIFg-1               | 3.3±3.3**abcB** | 26.7±6.7**bcD** | 33.3±8.8**bcD** | 60.0±10.0**cDA** |
| *Paecilomyces fumosoroseus* | Priority*             | 13.3±3.3**abcB** | 36.7±6.7**abA** | 46.7±8.8**bcA** | 63.3±3.3**abcD** |
| *Verticillium lecanii* | Nibortem®            | 6.7±3.3**abcC** | 26.7±3.3**bcD** | 40.0±10.0**cB** | 60.0±5.7**bcA** |
| *Beauveria bassiana*   | Nostalgist®          | 10.0±0.0**abcC** | 26.7±6.7**bcD** | 33.3±3.3**bcB** | 56.7±3.3**cDA** |
| Control (dH water+Tween-20) | 0.0±0.0**a**           | 0.0±0.0**a** | 0.0±0.0**a** | 0.0±0.0**a** |

*In single dose trial, all the isolates and products were tested at a concentration of 1 x 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^dC   | 13.3±3.3^dEBC | 36.7±8.8^AB    | 50.0±5.7^dA  |
|                  | B. bassiana          | 13.3±3.3^dC   | 20.0±5.7^dEBC | 36.7±3.3^dE    | 63.3±3.3^dA  |
|                  | B. bassiana          | 16.7±6.7^bcdeB | 33.3±3.3^bcdeB | 40.0±5.7^bcdeB | 60.0±5.7^dA  |
|                  | B. bassiana          | 36.7±3.3^dC   | 53.3±6.7^abcD | 76.7±3.3^ab    | 100±0.0^cA   |
|                  | B. bassiana          | 6.7±3.3^dC   | 16.7±3.3^dEBC | 30.0±5.7^dE    | 56.7±6.7^dA  |
|                  | B. bassiana          | 23.3±3.3^abcD | 43.3±3.3^abcD | 76.7±3.3^aB    | 100±0.0^cA   |
|                  | B. bassiana          | 13.3±3.3^dC   | 20.0±5.7^dEBC | 33.3±3.3^ab    | 56.7±3.3^dA  |
|                  | B. bassiana          | 33.3±3.3^abd  | 60.0±0.0^aC   | 73.3±3.3^aB    | 96.7±3.3^dA  |
|                  | B. bassiana          | 3.3±3.3^dE   | 20.0±5.7^dEBC | 26.7±6.7^dCAB  | 46.7±3.3^dA  |
|                  | B. bassiana          | 6.7±3.3^dE   | 20.0±5.7^dEBC | 33.3±6.7^dE    | 50.0±11.5^cDA|
|                  | B. bassiana          | 13.3±3.3^dE   | 23.3±6.7^dEBC | 33.3±6.7^ab    | 53.3±12^dA   |
|                  | B. bassiana          | 6.7±3.3^dE   | 13.3±3.3^dEBC | 23.3±3.3^cAB   | 33.3±3.3^dA  |
|                  | B. bassiana          | 0.0±0.0^C    | 6.7±3.3^EBC   | 16.7±3.3^dE    | 43.3±3.3^dA  |
|                  | B. bassiana          | 6.7±3.3^dE   | 10.0±5.7^EBC  | 23.3±6.7^E    | 60.0±10^dA   |
| Clonostachys rosea | CrMg-1             | 3.3±3.3^E   | 13.3±3.3^dEBC | 20.0±0.0^EBC  | 56.7±3.3^dA  |
|                  | C. rosea            | 6.7±3.3^dE   | 16.7±3.3^dE   | 36.7±3.3^aB    | 50.0±0.0^cA  |
| Isaria farinosa   | IfGp-1             | 3.3±3.3^E   | 10.0±0.0^EBC  | 20.0±5.7^E    | 50.0±5.7^aD  |
| Paecilomyces fumosorosus | Priority*  | 16.7±3.3^dEbcD | 23.3±3.3^dEbcD | 33.3±8.81^bab | 56.7±8.8^dA |
| Verticillium lecanii | Nibortem*       | 10.0±5.7^dEcd | 16.7±3.3^dEcd | 36.7±3.3^ab    | 66.7±3.3^cA  |
| Beauveria bassiana | Nostalgist*      | 20.0±0.0^abcd | 23.3±3.3^dEcd | 43.3±3.3^aB    | 63.3±3.3^dA  |
| Control (dH water+Tween-20)       | 0.0±0.0^A | 0.0±0.0^A | 0.0±0.0^A | 0.0±0.0^A |

*In single dose trial, all the isolates and products were tested at a concentration of 1 x 10^6 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  | JK110368.1    |
| TF6-1B       | B. bassiana   | ITS  | JK122736.1    |
| 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. brongniarti | ITS  | JX110385.1    |
| FUM03        | B. varroae    | ITS  | MF667767.1    |
| B5           | B. varroae    | ITS  | MH374536.1    |
| SASRI        | B. brongniarti | ITS  | JX110388.1    |
| ARSEF 2641   | B. amorpho    | ITS  | HQ880808.1    |
| BS18a        | B. amorpho    | ITS  | HQ880806.1    |
| BYYC-05      | B. asiatica   | ITS  | MG345071.1    |
| BU8824       | B. asiatica   | ITS  | MG642836.1    |
| ARSEF 4622   | B. australis  | ITS  | HQ880790.1    |
| ARSEF 4598   | B. australis  | ITS  | HQ880789.1    |
| FS85         | B. caledonica | ITS  | DQ529233.1    |
| BG47         | B. caledonica | ITS  | MT180427.1    |
| 1717         | B. vermicona  | 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 biopesticides 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 \textit{B. bassiana} isolate could be effective biocontrol agent against \textit{S. oryzae}. In another study by Komaki et al. (2017), seven EPF isolates (\textit{B. bassiana} (ARSEF-4984); \textit{Paecilomyces farinosus} (ARSEF-2538); \textit{Isaria fumosorosea} (ARSEF-4501); \textit{I. farinosa} (ARSEF-3580); \textit{Lecanicillium muscarium} (ARSEF-972 and ARSEF-5128), Mycotal extract of \textit{L. muscarium} (as positive control) and distilled sterile water with Tween-20 (as negative control) were tested against \textit{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 \textit{T. confusum} adults treated with seven EPF isolates varied from 34.6 to 100% after 10 days of exposure. The highest mortalities of \textit{T. confusum} adults were observed for \textit{P. farinosus} (ARSEF-2538) with 100% mortality at 1 × 10^7 conidia/ml and \textit{I. farinosa} (ARSEF-3580) with 97.3% mortality at 1 × 10^7 conidia/ml, followed by \textit{I. fumosorosea} (ARSEF-4501), \textit{B. bassiana} (ARSEF-4984) and \textit{L. muscarium} (ARSEF-5128) with 94.6% mortality. Unlike their findings, we found that \textit{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 \textit{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 \textit{S. oryzae} and \textit{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 \textit{S. oryzae} and \textit{T. confusum} in their prepared suspensions.

Finally, the present study revealed that some indigenous strains of soil-borne \textit{B. bassiana} were more effective to adult \textit{S. oryzae} and \textit{T. confusum} than the foreign origin commercial EPF products tested. Our results also indicated that the use of EPF, in particular indigenous strains of \textit{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 \textit{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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References

Adane, K., & Moore, D. S. A. (1996). Archer preliminary studies on the use of Beauveria bassiana to control Sitophilus zeamais (Coleoptera: Curculionidae) in the laboratory. Journal of Stored Products Research, 32, 105–113.

Alramadan, Y., & Mamay, M. (2019a). The importance of entomopathogenic fungi in the control of agricultural pests and promising fungal entomopathogens in the field Application. IGAC-2019. 1st International Gobeklitepe Agriculture Congress, (pp. 266-274), 25-27 November, Şanliurfa, TURKEY.

Alramadan, Y., & Mamay, M. (2019b). The importance of entomopathogenic bacteria in the control of agricultural pests and promising these entomopathogens in the field. IGAC-2019. 1st International Gobeklitepe Agriculture Congress, (pp.258-265), 25-27 November, Şanliurfa, TURKEY.

Alramadan, Y., & Mamay, M. (2019c). What is the role of entomopathogenic viruses in the control of agricultural pests and their future in the field application? IGAC-2019. 1st International Gobeklitepe Agriculture Congress, (pp. 301-309), 25-27 November, Şanliurfa, TURKEY.

Alramadan, Y., & Mamay, M. (2019d). The importance of entomopathogenic nematode and their role in the control of agricultural pests. IGAC-2019. 1st International Gobeklitepe Agriculture Congress, (pp.301-309), 25-27 November, Şanliurfa, TURKEY.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Research, 25, 3389–3402.

Barra, P., Rosso, L., Nesci, A., & Etcheverry, M. (2013). Isolation and identification of entomopathogenic fungi and their evaluation against Tribolium confusum, Sitophilus zeamais, and Rhyzopertha dominica in stored maize. Journal of Pest Science, 86, 217–226.

Batta, Y. A., & Abu-Safia, D. I. (2005). A study of treatment effect with Metarhizium anisopliae and four types of dusts on wheat grain infestation with red flour beetles (Tribolium castaneum) Herbst, Coleoptera: Tenebrionidae. Journal of the Islamic University of Gaza, 13, 11–22.

Batta, Y. A. (2004). Control of rice weevil (Sitophilus oryzae L., Coleoptera: Curculionidae) with various formulations of Metarhizium anisopliae. Crop Protection, 23, 103–108.

Batta, Y. A. (2008). Control of main stored-grain insects with new formulations of entomopathogenic fungi in diatomaceous earth dusts. International Journal of Food Engineering, 4, 1556–3758.

Batta, Y. A., & Kavalleratos, N. G. (2018). The use of entomopathogenic fungi for the control of stored-grain insects. International Journal of Pest Management, 64(1), 77–87.

Bidocha, M. J., Kamp, A. M., & De Croos, J. N. A. (2000). Insect Pathogenic Fungi: From Genes to Populations. In Fungal Pathology; Springer: Heidelberg, The Netherlands.

Boyer, S., Zhang, H., & Lempérière, G. (2012). A review of control methods and resistance mechanisms in stored-product insects. Bulletin of Entomological Research, 102(2), 213–229.

Campolo, O., Giunti, G., Russo, A., & Palmeri, V. (2018). Essential oils in stored product insect pest control. Journal of Food Quality, Article ID 6906105 (18pp.).

Cherry, A. J., Abalo, P., Hell, K., & Korie, S. (2007). Farm-scale trials to compare the entomopathogenic fungus Beauveria bassiana with pirimiphos methyl + deltamethrin and essential oil of lemon grass for protection of stored cowpea against Callosobruchus maculatus (Coleoptera: Bruchidae). Annals of Applied Biology, 152, 1–10.

Dal Bello, G., Padin, S., López, L. C., & Fabrizio, M. (2000). Laboratory evaluation of chemical-biological control of the rice weevil (Sitophilus oryzae L.) in stored grains. Journal of Stored Products Research, 37, 77–84.

Doyle, J. J., & Doyle, J. L. (1990). Isolation of plant DNA from fresh tissue. Focus, 12, 13–15.

Er, M. K., Tunaz, H., Ucuk, C., Baris, C., & Isikber, A. A.
(2016). Occurrence of entomopathogenic fungi on insect pests of stored wheat and maize in Central and South Anatolia in Turkey. *Türkçe Entomoloji Dergisi*, 40(3), 249–263.

Erler, F. (2005). Fumigant activity of six monoterpenoids from aromatic plants in Turkey against the two stored-product insects confused flour beetle, *Tribolium confusum*, and Mediterranean flour moth, *Ephestia kuehniella*. *Journal of Plant Diseases and Protection*, 112(6), 602–611.

FAO, (Food and Agriculture Organization of the United Nations) (2012). Second Global Conference on Research for Agricultural Development – Breakout Section: National Food Security – The Wheat Initiative. Available online at: http://www.fao.org/docs/eims/upload//306175/Brief%20Paper%20(3)-Wheat%20Initiative%20-%20H%C3%A9%20%20Lucas.pdf

Gaessler, G. (2014). The Value of Grains in a Healthful Diet. Available online at: http://gowiththegrain.org/pdf/TheValueofGrains.pdf

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98 NT. *Nucleic Acids Symposium Series*, 41, 95–98.

Howe, R. W. (2008). The effects of temperature and humidity on the rate of development and the mortality of *Tribolium confusum* Duval (Coleoptera, Tenebrionidae). *Annals of Applied Biology*, 48(2), 363–376.

Kassaye, A. (2010). *Susceptibility of the rice weevil, Sitophilus oryzae* (Coleoptera: Curculionidae) to native entomopathogenic fungal isolates. Addis Ababa University, Department of Biology, M.Sc. thesis, 51p, Ethiopia.

Kavallieratos, N. G., Athanassiou, C. G., Aountala, M. M., & Kontodimas, D. C. (2014). Evaluation of the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae*, and *Isaria fumosorosea* for control of *Sitophilus oryzae*. *Journal of Food Protection*, 1, 4–17.

Khashaveh, A., Ghosta, Y., Safaraziladeh, M. H., & Ziaee, M. (2011). The use of entomopathogenic fungus, *Beauveria bassiana* (Bals.) Vuill. In assays with storage grain beetles. *Journal of Agricultural Science and Technology*, 13, 35–42.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, 16, 111–120.

Komaki, A., Kordali, S., Usanmaz, B. A., Altınok, H., Kesdek, M., Simsek, D., & Altınok, M. (2017). Laboratory assessment for biological control of *Tribolium confusum* du Val., 1863 (Coleoptera: Tenebrionidae) by entomopathogenic fungi. *Türkçe Entomoloji Dergisi*, 41(1), 95–103.

Lacey, L. A. (2017). *Microbial Control of Insect and Mite Pests: From Theory to Practice*. Lawrence A. L. (ed.), 1st ed. (p. 461). Academic Press, Amsterdam, The Netherlands.

Lord, J. C. (2009). Efficacy of *Beauveria bassiana* for control of *Tribolium castaneum* with reduced oxygen and increased carbon dioxide. *Journal of Applied Entomology*, 133, 101–107.

Maina, U. M., Galadima, I. B., Gambo, F. M., & Zakaria, D. (2018). A review on the use of entomopathogenic fungi in the management of insect pests of field crops. *Journal of Entomology and Zoology Studies*, 6, 27–32.

Mamay, M., Ünlü, L., Yank, E., Doğramacı, M., & İkinci, A. (2016). Efficacy of mating disruption technique against carob moth, *Aponyelos ceratoniae* Zeller (Lepidoptera: Pyralidae) in pomegranate orchards in Southeast Turkey (Şanlıurfa). *International Journal of Pest Management*, 62(4), 295–299.

Mamay, M., & Dağ, E. (2016). Efficacy of Mass Trapping Technique against Carob Moth [*Aponyelos (= Ectomyelois) ceratoniae* Zell. (Lepidoptera: Pyralidae)] in Pomegranate Orchards. *II. International Multidisciplinary Congress of Eurasia*, (pp. 36-41), 11-13 July, Odessa, Ukraine.

Mamay, M., & Mutlu, C. (2019a). Trend biotechnological management methods against agricultural pests: mating disruption, mass trapping and attract & kill. IGAC-2019. 1st International Gokeblelkepe Agriculture Congress, (pp. 511-517), 25-27 November, Şanlıurfa, TURKEY.

Mamay, M., & Mutlu, C. (2019b). Optimizing container size and rearing density for rapid and economic mass rearing of *Oenopia conglobata* (Linnaeus, 1758) (Coleoptera: Coccinellidae). *Türkçe Entomoloji Dergisi*, 43(4), 395–408.

Mar, T. T., Suwannarach, N., & Lumnug, S. (2012). Isolation of fungi from Northern Thailand and their production in cereal grains. *World Journal of Microbiology and Biotechnology*, 28, 3281–3291.

Miller, D. W. (1995). *Commercial Development of Entomopathogenic Fungi: Formulation and Delivery*. (pp. 213–220). In Abstracts of Papers of the American Chemical Society; American Chemical Society: Washington, DC, USA.

Moino, J. A., Alves, S. B., & Pereira, R. M. (1998). Efficacy of *Beauveria bassiana* (Bal) Vuillemin isolates for control of stored grain pests. *Journal of Applied Entomology*, 122, 301–305.

Odour, G. I., Smith, S. M., Chandli, E. A., Karanja, L. W., Agano, J. O., & Moore, D. (2000). Occurrence of *Beauveria bassiana* on insect pests of stored maize in Kenya. *Journal of Stored Products Research*, 36, 177–185.

Padin, S., Dal Bello, G., & Fabrizio, M. (2002). Grain loss caused by *Tribolium castaneum*, *Sitophilus oryzae* and Acanthoscelides obtectus in stored durum wheat and beans treated with *Beauveria bassiana*. *Journal of Stored Products Research*, 38, 69–74.

Pimentel, M., Faroni, L., Batista, M., & Silva, F. (2008). Resistance of stored-product insects to phosphine. *Pesquisa Agropecuária Brasileira*, 43(12), 1671–1676.

Rajendran, S. (2002). *Postharvest Pest Losses*. (pp. 654–656). Encyclopedia of Pest Management, Pimentel, D. (Ed.). Marcel Dekker, Inc., New York.

Sheeba, G., Seshadri, S., Raja, N., Janarthanan, S., & Ignacimuthu, S. (2001). Efficacy of *Beauveria bassiana* for control of the rice weevil *Sitophilus*
oryzae (L.) (Coleoptera: Curculionidae). *Applied Entomology and Zoology*, 36, 117–120.

Stephou, V. K., Tjamos, S. E., & Paplomatas, E. J. (2012). Transformation and attachment of *Beauveria bassiana* conidia on the cuticle of *Tribolium confusum* and *Sitophilus oryzae* in conjunction with diatomaceous earth. *Journal of Pest Science*, 85, 387–394.

Talukder, F. (2009). Pesticide resistance in stored-product insects and alternative biorational management: a brief review. *Journal of Marine Science and Technology*, 14, 9–15.

Tamura, K. (1992). Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. *Molecular Biology and Evolution*, 9, 678–687.

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22, 4673–4680.

Tunc, I., Berger, B. M., Erler, F., & Dagli, F. (2000). Ovicidal activity of essential oils from five plants against two stored-product insects. *Journal of Stored Products Research*, 36(2), 161–168.

Veraverbeke, W. S., Delcour, J. A. (2002). Wheat protein composition and properties of wheat glutenin in relation to breadmaking functionality. *Critical Reviews in Food Science and Nutrition*, 42, 179–208.

White, T. J., Bruns, T., Lee, S., & Taylor, J. W. (1990). *Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes For Phylogenetics*. (pp. 315–322). PCR Protocols: a Guide to Methods and Applications, Academic Press, New York.

Yıldırım, E., Özbek, H., Aslan, İ. (2001). Stored products pests. *Atatürk Üniversitesi Ziraat Fakültesi Dergisi*, No: 191, 177 s., Erzurum