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ISOLATION OF ENTOMOPATHOGENIC FUNGI FROM TURKEY SOIL AND TESTING OF DIFFERENT DOSES ON *GALLERIA MELLONELLA* (LEPIDOPTERA: PYRALIDAE)

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ABSTRACT. Isolation of biological control agents which would be used with pests control could be done with different methods. The most sensitive of these methods is the insect bait method. Besides, it is known that the effects of different entomopathogenic fungi isolates, which would be used as biological control agents varies in different hosts. In this study, since the target pest organism is the larval period of *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidae), this pest is used for insect bait method and as a result of isolation from 180 soil samples, 48 *Beauveria bassiana* Bals.-Criv. Vuill. (Hypocreales: Cordycipitaceae) and 1 *Beauveria brongniartii* (Sacc.) Petch (Hypocreales: Cordycipitaceae) was detected but *Metarhizium spp.* wasn't detected. Furthermore, it is known that the precise determination of the entomopathogenic fungi isolates, plays a crucial role in the success of a biological control program. In this study, as biological control agent, four concentrations ($1x10^4$, $1x10^5$, $1x10^6$ and $1x10^7$ conidia/ml) of 10 different isolates of *Beauveria bassiana* Bals.-Criv. Vuill. (Hypocreales: Cordycipitaceae), which were obtained from soils of our country and a standard strain from Denmark were applied to *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidae) larvae. When Lethal times (LT_{50}) compared, it was found that the highest concentration of each isolates was also the most effective one.

1. INTRODUCTION

Due to the growing world population, natural ecosystems are being rapidly altered to acquire more agricultural land for human use. As an outcome the damage on natural ecosystems increase. The commercial and permanent farming system used

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for the production of sufficient nutrients should be both highly efficient and sustainable additionally should have minimal environmental pollution. On the other hand, natural life needs to be preserved. Achieving both goals is one of the most important problems of the 21st century. One of these solutions is biological control [1]. Chemical pesticides are used in the control of many harmful insects [2] and have proven to be effective in many cases [3]. They are particularly effective in controlling weed and plant diseases. However, they are ineffective in controlling certain insect such as silkworms [4,5]. As insects develop resistance to the chemicals used [6]. Moreover, because of the degree of pollution caused by chemicals, methods of fighting insects that are less harmful to both the environment and humans are having been developed [1,7,8,9,10]. At this stage, the idea of biological control was born, and attempts were made to prevent pests by using various living organisms [11,12,13]. Various living groups, from the smallest microorganisms to vertebrates, are being used in biological control including bacteria, fungi, nematodes, birds and insects [14]. Microbial biological control agents offer effective, environmentally harmless and cheaper long-term economic methods to deal with harmful insects [13,15]. Numerous entomopathogenic fungi are in use for the control of pests [16]. *Beauveria bassiana* Bals.-Criv. Vuill. (Hypocreales: Cordycipitaceae) and *Metarhizium anisopliae* (Metschn.) Sorokin (Hypocreales: Clavicipitaceae) are the most studied entomopathogenic fungi species, both having very wide host range [17,18] and have wide geographical distribution [15]. There are numerous successful biological control experiments carried with *B. bassiana* [19,20]. This species is known to be able to infect more than 200 species of insects [21,22]. It was reported to be the most common and most effective fungus against ground beetles that can survive the winter [21,22,23].

It was stated that the effects of isolates obtained from different regions in different hosts might be different [24,25]. In addition, accurate determination of the concentration to which entomopathogenic fungus would be administered was an important factor affecting its success as a control agent. [26,27] In many studies, it was stated that the entomopathogenic fungus isolated from the area to be applied would be more successful in that area than the entomopathogenic fungus isolated from another region. [24,25,27,28]. Therefore, this study was designed to investigate the effects of different strains and concentrations of *B. bassiana* and *M. anisopliae* using *Galleria mellonella* larvae, which were preferred as experimental animals in many studies [29]. However, since there was not any *M. anisopliae* species in our isolation studies, our studies were conducted on *B. bassiana* strains. In this study, four different concentrates for each of 10 strains of *B. bassiana* isolated from various regions of Turkey with the standard strain of *B. bassiana*, whose pathogenity was proven on insects, were tested on the larvae of *G. mellonella*.

2. Materials and Methods

2.1. Galleria mellonella

The reproduction and development of *G. mellonella* larvae used in this study was carried out in glass jars and in a dark artificial medium environment in the laboratory with a 27 °C temperature, which is suitable environment for larvae [30]. *Galleria* larvae emerging from their eggs after 4 weeks (fourth stage of larvae) was used for the insect bait method [31]. During the insect bait method, *Galleria* larvae were kept in 500 ml of water which was heated up to 56 °C to prevent larvae from pupation and their inactivation was fulfilled [31,32,33].

2.2. Collection of soil samples, insect-bait and soil dilution method

During May and August of 2007-2008, a total of 180 soil samples were collected from soils around Turkey and the points where soil samples were taken were randomly selected [34]. Soil samples of approximately 1 kg were collected from cultivated areas, meadows and forest floors. Soil samples were preferably taken from the surface of the soil from 5 - 20 cm depth [21,35,36,37,38,39,40]. These samples were taken into plastic bags to prevent drying of the soil after the labeling process, where values such as the name of the regions, vegetation type and soil temperature were recorded [21,36,41].

The insect-bait method was originally developed for isolation of entomopathogenic nematodes found in soil, but this method also provides isolation of entomopathogenic fungi [42]. The insects to be used in this method should be easily produced and that are sensitive to fungi should be preferred. The most common insect species used for this purpose is the big wax moth *G. mellonella* [31,32,33]. In the isolation of entomopathogenic fungi, baiting of soil samples with *Galleria* larvae is a very common tool [21,36,37,39,43,44,45].

Soil samples obtained from the different field were taken into 250 ml volume plastic containers without waiting too long. In dry soil samples, distilled water was added, considering that for entomopathogenic fungi infection is better in moist conditions. Inactivated fourth stage *Galleria* larvae were placed in five of the soil samples that are placed in plastic containers [46]. After the mouth of the plastic containers was closed, the plastic containers were inverted so that *Galleria* larvae remained under

the soil [40,47,48]. The samples were kept at room temperature at 20-25 °C [46,49]. The soil samples, which were inverted, were checked for a total of 15 days each 3 days apart to see if the larvae were infected [50].

The larvae thought to be infected were removed from the soil to provide surface sterilization for the cadaver with 1% sodium hypochlorite in 2-3 minutes, then washed with distilled water [31,43,51,52,53,54]. Then the larvae were transferred to sterile petri dishes with damp filter paper in them [55,56]. With daily checks, the larvae were investigated whether the cause of death was entomopathogenic fungus [56,57]. At the same time, distilled water was added to the filter paper in the petri dishes at regular intervals due to the high humidity requirement of entomopathogenic fungi (Figure 1) [56,58,59,60].

FIGURE 1. *Galleria* larvae infected with *Beauveria bassiana* and *Beauveria bassiana* spores that grow on the larvae.

When entomopathogenic fungi started to produce conidia on cadavers and, the samples that taken here were added to the selective media [59]. Fungi thought to be entomopathogen were placed on the media containing SDA + dodine + chlorotetracycline (prepared by adding 0,46 g/l dodine and 5 mg/l chlortetracycline to SDA media) and procreation of entomopathogenic fungi was provided after 7 days of incubation at 27 °C [61,62, 63,].

Soil dilution method, another isolation method of entomopathogenic fungi from soil, was also carried. Soil samples collected from various regions of Turkey were placed

in sterile tubes containing 10 ml distilled water. Each test tube was mixed in centrifuge for 20 seconds. Then 1 ml was taken from the mixture and added to the test tubes with 9 ml distilled water and thus 10 times dilution was achieved [38,60,74]. Then, a mixture of 0.5 ml from each test tube was added to Veen's Medium, the selective medium of entomopathogenic fugus, and spread with the help of a sterile L glass rod. Media were left to fungal development for 14 days at 26°C ± 2°C. Afterwards, samples that may be *Beauveria spp.* and *Metarhizium spp.* which reproduce in media were planted in SDAY media to obtain pure culture [64].

2.3. Selection of experimental strains

The fungus samples isolated by both methods were selected according to the colony characteristics in SDAY media in the first stage. Considering the colonial characteristics of the species that may be *B. bassiana* and *Metarhizium anisopliae* (Metschn.) Sorokin (Hypocreales: Clavicipitaceae), the specimens that did not provide these characteristics were eliminated. At the begining the color of *B. bassiana* colonies were white and then go to yellowish pinkish [65,66,67], while the color of *M. anisopliae* colonies were yellow to green or dark plant green to pink [66,68].

In order to distinguish the entomopathogens from the selected samples, the fungi were planted in PDA media [69]. Cultures were incubated at 27 °C for 14 days so that they can produce conidia [15]. Then five *Galleria* larvae were left in these cultural environments and the larvae were expected to wander in the petri dish for a certain period of time, allowing them to take conidia on them [70]. In the second stage, these larvae were taken from the culture environment and placed in petri dishes with sterile filter papers [60]. Drying papers were moistened with sterile distilled water to provide moisture for fungal growth [66,71]. The conditions of the larvae were observed every day and checked whether the cause of death was the fungus. The larvae infected with entomopathogenic fungi were recorded, while others were eliminated.

2.4. Obtaining entomopathogenic fungus species

Spores taken from larvae infected with entomopathogenic fungus were placed in SDA media with 1 ml dodine (prepared by dissolving 1 gr dodine in 9 ml distilled water solution) and 500 µl chloramphenicol (prepared by dissolving 1 gr chloramphenicol in 10 ml of a 96% solution of ethanol) added and incubated at 26°C

± 2°C. After 7-14 days of incubation, entomopathogenic fungus cultures that were freed from soil bacteria and saprophytic fungi were obtained [72,73].

2.5. Preparing slide and microscopic examination

Entomopathogenic fungus strains were placed in SDAY media and preparations of entomopathogenic fungus strains were made and examined under a microscope [65,74]. In this microscopic examination, the elimination of non-entomopathogenic fungi by taking into account several key features of entomopathogenic fungi. During the preparation of the slide, very small amounts of fungal samples were taken from the culture medium where the entomopathogenic fungus had been produced. Then the previously prepared lactophenol solution was dripped on microscope slide in a small drop and the sample was left on it. Entomopathogenic fungus samples taken from the medium with the inoculating loop, the medium particles on the inoculating loop were dissolved without getting too close to the bunsen flame. Thus, entomopathogenic fungi spread on microscope slide without damaging its structure and a cover glass was placed on it and examined under a microscope.

On prepared slide, the genus *Beauveria* tried to be observed the most characteristic features of the indicated conidiogenous cells densely clustered or in whorls or solitary, colorless, short, with base globose or flask-like and extending apically and repeatedly branching a short distance below each of several apically-formed conidia (Figure 2) and as for that the genus *Metarhizium* tried to be observed on prepared slide, such as conidiogenous cells with rounded to conical apices, arranged in dense hymenium; conidia aseptate, cylindrical or ovoid, forming chains usually aggregated into prismatic or cylindrical columns or a solid mass of parallel chains, pale to bright green to yellow-green, olivaceous, sepia or white in mass [65,75,76].

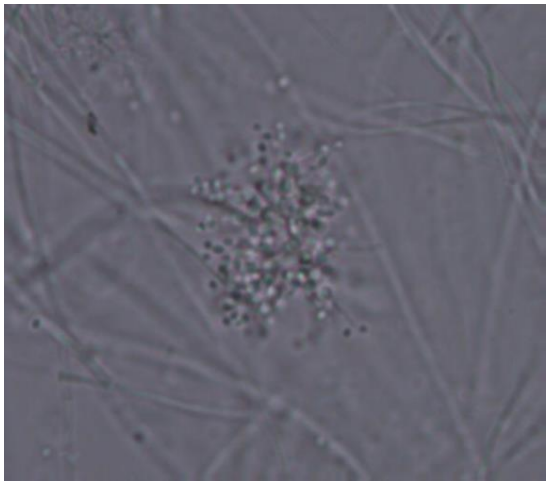

FIGURE 2. It was tried to observe that *B. bassiana* conidiaphore, sporogenic cell and conidias.

The absolute species identification of entomopathogenic fungus samples, which were isolated from soil by using insect bait method and soil dilution method and thus removed from saprophytic fungi and bacteria using selective media.

2.6. Used experimental strains

Until this stage of our studies, 48 strains of *B. bassiana* and 1 strain *Beauveria brongniartii* (Sacc.) Petch (Hypocreales: Cordycipitaceae) were identified in consequence of isolation and species identification from soil samples collected from various regions of Turkey. 48 *B. bassiana* strains that obtained from isolation were tested on *Galleria* larvae and 10 *B. bassiana* strains with the best pathogenity were selected. The type species is KVL 03-129 *B. bassiana* 2002 standard strain from Copenhagen University, Denmark. The locations where these 10 examples were taken and the vegetation types of these places are listed in Table 1.

TABLE 1. *Beauveria bassiana* strains, vegetation type of the place and area where it was isolated.

	Code Of The Strain	Where soil sample was taken	Type of vegetation where soil sample was taken
1. Strain	DenK1	Denizli - Kocabey	Plantation
2. Strain	SB1(b)	Ankara - Beytepe	Meadow Field
3. Strain	Ank 12	Ankara - Çalış Köyü	Plantation
4. Strain	Lül 5	Tekirdağ - Lüleburgaz	Tree Planted Area
5. Strain	MerK	Mersin - Kargıpınar	Plantation
6. Strain	BarK2	Bartın - Kumluca	Woodland
7. Strain	Kon-2	Konya - Cihanbeyli	Plantation
8. Strain	Ordu	Ordu - Merkez	Hazelnut Tree Gardens
9. Strain	AyAk	Aydın - Akbük	Olive Tree Gardens
10. Strain	Erz	Erzincan - Tercan, Aşkale	Meadow Field

2.7. Preparation of spore solutions

The spore solution of each strain was prepared from cultures that had been produced for 14 days in SDAY media that prepared in 50 ml tubes before the experimental setup was prepared. 10 ml distilled water containing 1% Tween 80 was added to these media. Then the conidia suspension of each strain was transferred into 50 ml volume tubes and mixed for 3 minutes at 1500 rpm to separate the spores and micelles from each other [68]. Conidial concentration in suspension was detected under phase contrast microscope using Neubauer chamber [66,73].

0.1 ml of spore solution was placed on Neubauer chamber which has four large squares, each 1 mm^2 in size. The number of spores in 1 mm^3 was determined by going into them by 4 in order to get an average of the number of spores in these four square areas. This number was then multiplied by 10^3, resulting in the number of sports in 1 ml. The number of spores was found by multiplying this number by the dilution factor [63].

In our study, 4 different concentrations were prepared for each fungus strain and standard strain and were tested on larvae in the experiments to test the 10 fungal strains whose pathogenity was to be tested on *G. mellonella* larvae. These concentrations were $1x10^4$, $1x10^5$, $1x10^6$ and $1x10^7$ conidia/ml [77].

2.8. Testing of fungal strains on *Galleria mellonella* larvae

4 different spore solutions ($1x10^4$, $1x10^5$, $1x10^6$ and $1x10^7$ conidia/ml) prepared for each selected strain of entomopathogenic fungus were added as 1 ml to the sterile petri dish containing sterile filter paper. 10 inactivated fourth stage instar *Galleria* larvae were placed in each petri dish. The dose of each entomopathogenic fungus strain was applied to 10 *Galleria* larvae and the dosage applications were repeated three times. Thus, 30 larvae were used for each dose. All these procedures were carried out in sterile conditions to prevent contamination.

In this experiment, each strain was put into plastic bags separately each as a group, thus preventing contamination and moisture loss and were removed to the environment where 27 °C and dark conditions were achieved. The established experiments were checked every day and the dead larvae found to be infected were soaked in 1% sodium hypochlorite for 2 minutes to ensure surface sterilization and then washed with distilled water and transferred to petri dishes containing sterile filter paper under sterile conditions. The moisture was provided by adding 1 ml of distilled water. These petri dishes were placed in separate plastic bags and checked daily for the observation of entomopathogenic fungi on cadavers in the same environment. Nevertheless, sterile distilled water has been added to these petri dishes to ensure the high humidity environment required for fungi. In addition, control groups were formed in the same way as the experiments, but the control group was not given spores and sterile distilled water was added in 1% Tween 80 [60].

2.9. Statistical methods

The pathogenity of these 10 strains, whether the dose applied and the effect of the strain were investigated by Anova based on the pathogenity of the type species. Groups (strains) were compared among themselves with LSD testing.
The EPA probit analysis program was used based on the killing potential of *Galleria* larvae of all fungal strains used in the experiment on the fifth day of the experiment, and thus LD_{50} (the dose required to kill 50% of the subjects) values were calculated. In addition, with the Kaplan-Meier test, LT_{50} values, in other words the time it took for 50% of *Galleria* larvae to die, and standard error (SE) were calculated [78].

3. RESULTS

Whether the dose and strain effects of these 10 strains were investigated with Anova according to the pathogenicity effects of the standard strain and the results indicated that these two factors were statistically significant. (F_{strain} = 2,06, F_{dose} = 9,30, P<0,05). The strains were compared among themselves with the LSD test (Table 2).

TABLE 2. Comparison of different doses of *Beauveria bassiana* strains which tested on *Galleria mellonella* with each other and with the control group.

Dose (con/ml)	Dose (con/ml)	Mean differences	Standard Eror	Sig.	95% Limits	Confidince
					Sub Limit	Upper Limit
$1x10^7$	$1x10^6$	,791	,774	,308	-,733	2,315
	$1x10^5$	1,871	,732	,011	,429	3,312
	$1x10^4$	2,310	,715	,001	,901	3,719
	C	4,609	,987	,000	2,666	6,552
$1x10^6$	$1x10^7$	-,791	,774	,308	-2,315	,733
	$1x10^5$	1,080	,702	,125	-,303	2,463
	$1x10^4$	1,519	,685	,027	,170	2,868
	C	3,818	,965	,000	1,918	5,718
$1x10^5$	$1x10^7$	1,871	,732	,011	-3,312	-,429
	$1x10^6$	1,080	,702	,125	-2,463	,303
	$1x10^4$	,439	,637	,491	-,815	1,694
	C	2,738	,932	,004	,903	4,572
$1x10^4$	$1x10^7$	2,310	,715	,001	-3,719	-,901
	$1x10^6$	-1,519	,685	,027	-2,868	-,170
	$1x10^5$	-,439	,637	,491	-1,694	,815
	C	2,298	,919	,013	,490	4,107
C	$1x10^7$	4,609	,987	,000	-6,552	-2,666
	$1x10^6$	3,818	,965	,000	-5,718	-1,918
	$1x10^5$	2,738	,932	,004	-4,572	-,903
	$1x10^4$	2,298	,919	,013	-4,107	-,490

A high rate of successful mortality was obtained by comparing all fungal strains and their doses with the control group on *Galleria* larvae. The results of the statistical analysis also supported this result and it was found that the average death time of the control group according to LSD test was different from the average death time of all tried doses (P<0,05) (Table 2).

It was found that there was no difference between the results of concentration applications of fungus strains $1x10^7$ and $1x10^6$ con/ml ($P>0.05$) (Table 2) also there was no difference in concentration applications of fungi strains $1x10^6$ to $1x10^5$ and $1x10^5$ to $1x10^4$ con/ml ($P>0.05$) (Table 2). It was found that the concentration application of $1x10^7$ con/ml was different from $1x10^5$ and $1x10^4$ con/ml, and the concentration application of $1x10^6$ con/ml was different from $1x10^4$ con/ml ($P<0.05$) (Table 2). According to these results, it was found that the effective dose in terms of efficacy (the dose that killed in the shortest time) was the highest of the applied doses, and the effectiveness decreased with respect to the high dose as the applied dose decreased. This result was supported by both graphs and statistical results (Figure 3) (Table 2). In this study, Erz strain was found to be most effective compared to other strains according to average death times and graphs (Table 3) (Figure 2).

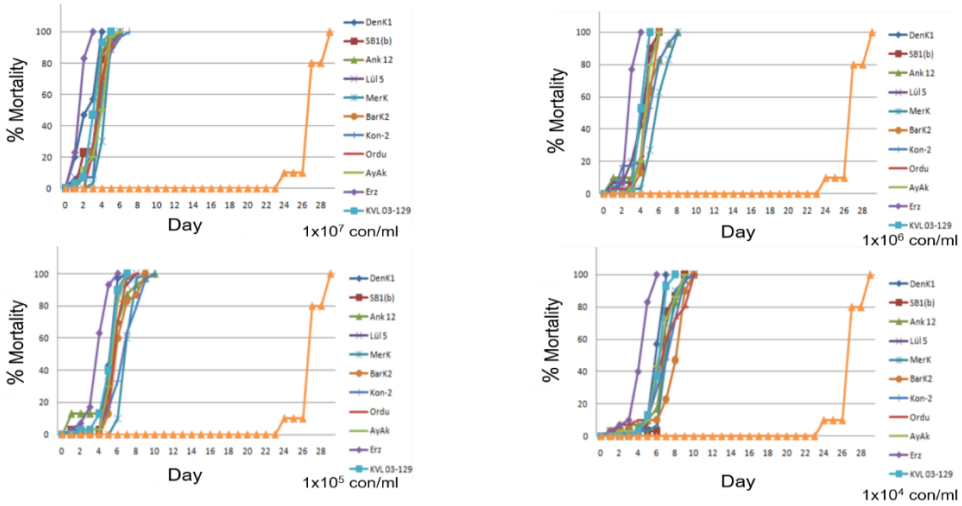

FIGURE 3. It was observed that different doses of 10 *Beauveria bassiana* strains and KVL 03-129 which was a type species, to *Galleria mellonella* on time-dependent mortality rate graphic**.**

TABLE 3. Average death times of used different doses of *Beauveria bassiana* strains and KVL03-129 which was a type species, applied to *Galleria mellonella* larvae.

Fungal Strain	Applied Dose (con/ml)	Average death times (day)	Standard Deviation
DenK1	$1x10^7$	2,80	1,22
	$1x10^6$	4,30	0,92
	$1x10^5$	5,52	0,85
	$1x10^4$	6,33	0,66
SB1(b)	$1x10^7$	3,70	1,02
	$1x10^6$	4,87	0,68
	$1x10^5$	5,71	1,04
	$1x10^4$	7,20	1,21
Ank 12	$1x10^7$	3,90	0,80
	$1x10^6$	4,83	1,78
	$1x10^5$	5,77	2,20
	$1x10^4$	7,10	1,85
Lül 5	$1x10^7$	3,97	1,10
	$1x10^6$	4,40	1,30
	$1x10^5$	5,70	1,06
	$1x10^4$	6,80	1,58
MerK	$1x10^7$	4,73	0,64
	$1x10^6$	6,13	1,41
	$1x10^5$	7,33	0,71
	$1x10^4$	7,44	1,45
BarK2	$1x10^7$	3,97	0,96
	$1x10^6$	5,20	0,76
	$1x10^5$	6,57	1,20
	$1x10^4$	8,07	1,32
Kon-2	$1x10^7$	4,40	1,13
	$1x10^6$	5,17	1,70
	$1x10^5$	6,97	1,77
	$1x10^4$	7,37	1,56
Ordu	$1x10^7$	4,17	0,91
	$1x10^6$	4,87	0,97
	$1x10^5$	6,03	1,07
	$1x10^4$	7,17	2,13
AyAk	$1x10^7$	4,20	1,10
	$1x10^6$	4,97	0,72
	$1x10^5$	5,80	0,85
	$1x10^4$	6,77	1,46
Erz	$1x10^7$	1,93	0,64
	$1x10^6$	3,17	0,53
	$1x10^5$	4,17	1,05
	$1x10^4$	4,57	1,14
KVL 03-129	$1x10^7$	3,55	0,85
	$1x10^6$	4,47	0,51
	$1x10^5$	5,50	1,04
	$1x10^4$	6,53	0,89
Control	0	27,1	1,32

TABLE 4. LT_{50} values of used different doses of *Beauveria bassiana* strains and KVL03-129 which was a type species, applied to *Galleria mellonella* larvae

Fungal Strain	Applied Dose (con/ml)	LT_{50} (day)*	Standard Eror	% 95 Confidince Limits
DenK1	$1x10^7$	3	0,15	2,69-3,30
	$1x10^6$	4	0,28	3,43-4,56
	$1x10^5$	6	0,06	5,88-6,12
	$1x10^4$	6	0,19	5,62-6,37
SB1(b)	$1x10^7$	4	0,22	3,55-4,44
	$1x10^6$	5	0,07	4,85-5,14
	$1x10^5$	6	0,20	5,83-6,16
	$1x10^4$	7	0,26	6,77-7,22
Ank 12	$1x10^7$	4	0,09	3,81-4,18
	$1x10^6$	5	0,35	4,29-5,70
	$1x10^5$	6	0,20	5,60-6,39
	$1x10^4$	7	0,26	6,48-7,51
Lül 5	$1x10^7$	4	0,21	3,58-4,41
	$1x10^6$	5	0,16	4,67-5,32
	$1x10^5$	6	0,10	5,80-6,19
	$1x10^4$	7	0,31	6,39-7,61
MerK	$1x10^7$	5	0,07	4,85-5,14
	$1x10^6$	6	0,23	5,53-6,46
	$1x10^5$	7	0,17	6,65-7,34
	$1x10^4$	8	0,13	7,74-8,25
BarK2	$1x10^7$	4	0,17	3,65-4,34
	$1x10^6$	5	0,17	4,65-5,34
	$1x10^5$	6	0,18	5,63-6,37
	$1x10^4$	9	0,12	8,75-9,24
Kon-2	$1x10^7$	4	0,35	3,30-4,70
	$1x10^6$	6	0,22	5,56-6,43
	$1x10^5$	7	0,28	6,43-7,56
	$1x10^4$	8	0,29	7,41-8,58
Fungal Strain	Applied Dose (con/ml)	LT_{50} (day)*	Standard Eror	% 95 Confidince Limits
Ordu	$1x10^7$	4	0,17	3,65-4,34
	$1x10^6$	5	0,12	4,74-5,25
	$1x10^5$	6	0,16	5,67-6,32
	$1x10^4$	7	0,36	6,27-7,72
AyAk	$1x10^7$	5	0,08	4,83-5,16
	$1x10^6$	5	0,12	4,76-5,23
	$1x10^5$	6	0,12	5,75-6,24
	$1x10^4$	7	0,26	6,48-7,51
Erz	$1x10^7$	2	0,11	1,77-2,22
	$1x10^6$	3	0,11	2,78-3,21
	$1x10^5$	4	0,18	3,63-4,36
	$1x10^4$	5	0,15	4,69-5,30
KVL 03-129	$1x10^7$	4	0,09	3,80-4,19
	$1x10^6$	4	0,19	3,62-4,37
	$1x10^5$	6	0,10	5,78-6,21
	$1x10^4$	7	0,08	6,84-7,15
Control	0	27	0,31	26,39 – 27,61

* The time it takes for 50% of the larvae to die

Moreover, LT_{50} values that obtained in the Kaplan-Meier test (Table 4) and LD_{50} values that obtained through the EPA probit analysis program (Table 5) support this result. Statistical analysis also showed that Erz was significantly different from Ank12, MerK and Kon - 2 strains ($P<0.05$), but not different from other strains ($P>0.05$).

TABLE 5. LD_{50} values of used *Beauveria bassiana* strains and KVL03-129 type species, were calculated according to their potential to kill *Galleria mellonella* larvae on the 5th day

Fungal Strain	LD_{50} (Killing Dose)	95% Confidince Limits
DenK1	$8,6x10^4$	$5,2x10^4$ - $1,4x10^5$
SB1(b)	$1,9x10^5$	$1,1x10^5$ - $3,1x10^5$
Ank 12	$2,8x10^5$	$1,4x10^5$ - $5,4x10^5$
Lül 5	$1,8x10^5$	$1x10^5$ - $3,3x10^5$
MerK	$1,7x10^6$	$9,2x10^5$ - $3,7x10^6$
BarK2	$3,4x10^5$	$1,5x10^5$ - $6,3x10^5$
Kon-2	$7x10^5$	$3,4x10^5$ - $1,5x10^6$
Ordu	$3,1x10^5$	$1,6x10^5$ - $6,1x10^5$
AyAk	$2x10^5$	$1,1x10^5$ - $3,8x10^5$
Erz	$6,5x10^2$	0,00 - $4,5x10^3$
KVL 03-129	$8,5x10^4$	$5x10^4$ - $1,4x10^5$

Within ten days, all strains caused high levels of white muscardine disease in every dose. LT_{50} values of Erz and DenK1 strains were found to be lower (effective in a shorter time) than standard strains and LT_{50} values of Merk and AyAk strains were found to be higher at a dose of $1x10^7$ con/ml. At a dose of $1x10^6$ con/ml, LT_{50} value of the Erz strain was also found to be lower than the standard strain. At this dose, the LT50 value of the DenK1 strain and the standard strain were the same, while other strains had a longer LT_{50} value than the standard strain. At this dose, the DenK1 strain had the same LT_{50} value as the standard strain, while other strains had a higher LT_{50} value than the standard strain. Kon-2 strain was one of the strains with the highest LT_{50} value at this dose. LT_{50} value of the Erz strain was low compared to the standard strain at dose of $1x10^5$ con/ml, while the Merk and Kon-2 strains were high. At dose of $1x10^4$ con/ml, LT_{50} value of Erz and Denk1 strains were low compared to the standard strains, while those of Merk and BarK2 and Kon-2 strains were high. It was found that the effectiveness of all fungal strains were not statistically different between them compared to the standard strains (Table 4) ($P>0.05$).

3. Discussion

Sensitive insects such as *G. mellonella* and *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrioidae) were used in isolation of entomopathogenic fungus strains from soil [29,31,32,45]. In our study, *G. mellonella* was preferred as the target organism due to the fact that *G. mellonella* was well known the parasite of bee's wax so it had more devastating economical effect for the beekeeping industry [46,79,80,81,82].
Insect bait method was first developed for detection of entomopathogenic nematodes using *Galleria* larvae in the soil by Akhurst *et al.* and then, Zimmermann (1986) and Mietkiewski *et al*. stated that this method was also successful in the isolation of entomopathogenic fungi [42,47,83,84]. Keller *et al.* (2003) stated in a study that this method was more effective [37]. Meyling (2007) stated that the insect bait method was more advantageous than the soil dilution method. He stated that entomopathogenic fungi became difficult to find due to the use of fewer soil samples in soil dilution method; the number of spores decreased further by dilution; and that the reproduction in the medium of other opportunistic saprophytic fungi put this method at a disadvantage. Using more soil in insect bait method made this method more selective for isolation of entomopathogens [33]. Kessler *et al.* (2004) also emphasized in their study that insect bait method was a more sensitive method than the soil dilution method [38]. Dhoj *et al.* (2008) attempted to isolate entomopathogenic fungi using the insect bait method and soil dilution method from 46 soil samples they collected from Nepal between 2002 and 2005. As a result, they isolated 78 entomopathogenic fungi samples by insect bait method and 5 entomopathogenic fungi samples by soil dilution method [50]. Our findings were similar to those of these researchers. On the other hand, Imoulan *et al.* (2019) and Gürlek *et al.* (2018) isolated entomopatojenic fungus by soil dilution method [85,86]. In our study, the number of fungi isolated by insect bait method was found to be greater than the number of fungi isolated by soil dilution method. Those findings and our result showed us that insect bait method could be more precise method.

The soil is the classical place of isolation for entomopathogenic fungi [45,86] and many entomopathogenic fungi can be found in both agricultural soil and natural habitats [21,37,44,87,88]. In this study, 48 *B. bassiana* and 1 strain *B. brongniartii* were isolated and *Metarhizium spp.* was not isolated. This result showed us that there was not *Metarhizium spp.* in the soils that we studied. In addition, Sahin (2006) stated

that he isolated 19 *B. bassiana* strains from soil samples collected from Kahramanmaraş province, but failed to isolate *Metarhizium spp.* [89]. Our findings were similar to those of these researcher. But, Sevim *et al.* (2010) stated that they isolated 62 entomopathogenic fungus strains from 301 soil samples they collected from their research sites by *Galleria* bait method and that of these 62 entomopathogenic fungi, the species of *Metarhizium* genus was the most abundant [90]. Moreover, Keppanan *et al*. (2018) stated that entomopathogenic fungi of the genus *Metharizium*, genus *Beauveria* and genus *Isaria* were isolated from agricultural areas of Thekkady region of India by the *Galleria* bait method [55].

In this study, 28 of the fungi were isolated from farmland samples while 21 were isolated from uncultivated soil samples. In other studies, entomopathogenic fungi were found in different habitats in different countries at different rates. Sevim *et al.* (2010) stated that they isolated entomopathonic fungus both from agricultural and nonagricultural soils [90]. Meyling and Eilenberg (2006) found that *B. bassiana* was widespread in soils in agricultural areas of Denmark, while *M. anisopliae* was rare [87]. This conclusion was consistent with our findings. However, Sun *et al*. (2008) showed that insect pathogen fungi were more likely to be found in orchard soils than in farmland soils, and Vanninen (1995) noted that in Finland, insect pathogen fungi were more likely to be found in forest soils than in agricultural soils [91,92]. Imoulan *et al.* (2019) found that *Beauveria* species were recovered more frequently from forested soils than from farmland [86]. As a result of all these studies, it was understood that entomopathogenic fungi may have different proportions in farmland and uncultivated soils.

The reason *B. bassiana* was more isolated in insect bait method than *B. brongniartii* may be that *B. bassiana* infected and killed larvae more quickly. Keller *et al.* (2003) noted that they isolated *M. anisopliae* strain more than *B. brongniartii* strains by insect bait method. They stated that the cause of this condition was *M. anisopliae* could kill *Galleria* larvae faster than *B. brongniartii* [37]. Also, Keppanan *et al*. (2018) found that *M. anisopliae* infected *Galleria* larvae used in the insect bait method faster than other entomopathogenic fungus species [31]. This findings may explain why *B. bassiana* was more isolated by insect bait method than *B. brongniartii* in our study. Chase *et al.* (1986) found that 0.55 g/l dodine in the medium only allowed optimal isolation of *B. bassiana*, while reducing the amount of dodine to 0.46 g/l allowed isolation of *B. bassiana* as well as other entomopathogenic fungal strains [62]. In this study, *B. brongniartii* was isolated alongside *B. bassiana* by adding 0.46 g/l dodine to the SDA medium and this result was in harmony with the findings of Chase *et al.*

In this study, isolated *B. bassiana* strains were tested on *Galleria* larvae before the experiment was initiated, and infective 10 fungal strains were selected from among these strains. In the experiment, 4 different doses of these strains and the type species were used, and a control group was formed to determine whether Tween 80 solution had an effect on the survival of *Galleria* larvae and to show that uninfected larvae were healthy compared to those exposed to the doses. The dose range used in such studies usually varies depending on the host and the manner of administration, but quantities between $1x10^4$ - $1x10^8$ con/ml were used for every 10 insects. Furthermore, in such experiments, the purpose of testing different doses on the target organism was to observe small changes in virulence [17,23,31,44,93,94].

The standard strain used in this study, KVL 03-129 (ARSEF 8032), was a strain from the ARS entomopathogenic fungus collection and was isolated from agricultural region soil using *Galleria* bait method by Nicolai V. Meyling on September 18, 2002. In this study, entomopathogenic fungus strains isolated from Turkey soil were isolated by the same method, resulting in a healthy result in pathogenicity experiments. Because in biological control studies, strains isolated from homologous hosts were more successful in using entomopathogenic fungi as a biological control agent [23,85,95,96]. In addition, while it was common to search for the same origin agent as the target organism in biological control studies, successful controls were also obtained with natural enemies that did not come from the natural area of the target pest [23,31,85]. Klingen *et al.* (2002) observed in a study that the *M. anisopliae* strain (ARSEF 5520) was highly pathogenic to *G. mellonella*, but was not pathogenic to *Delia floralis* (Fallen) (Diptera: Anthomyiidae) larvae, although it was an isolated strain using *D. floralis* in bait method [44].

All tried doses of these 10 strains and standard strains were found to be highly successful on *Galleria* larvae compared to the control group. Klingen *et al.* (2002) experimented with a dose of $1x10^7$ con/ml of 3 different strains of *B. bassiana* on *Mamestra brassicae* (Linnaeus) (Lepidoptera: Noctuidae) and indicated that these strains were highly successful compared to the control group [44]. Trudel *et al.* (2007) tested the doses of $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$, $1x10^8$, $1x10^9$ and $1x10^{10}$ con/ml of 6 different *B. bassiana* strains on *Pissodes strobi* Champion & G.C. (Coleoptera: Curculionidae) and obtained a successful mortality result according to the control group [16]. Özdemir *et al.* (2020) tested the dose of $1x10^6$ and $1x10^8$ con/ml of *B. bassiana* and *M. anisopliae* on *Callosobruchus maculatus F.* (Coleoptera: Chrysomelidae: Bruchinae) at two different tempreture (22 – 26 ^{0}C) [97]. They obtained a successful mortality result too. Our findings were similar to those of these researchers.

In this study, the highest mortality in the shortest period of time of all fungal strains tried on *G. mellonella* was achieved by the application of the highest dose ($1x10^7$ con/ml) of each entomopathogenic fungus strains. Kim and Kim (2008) experimented with 5 different doses of the *Lecanicillium attenuatum* Zare & W.Gams (Hypocreales: Cordycipitaceae) strain, was a entomopathogenic fungus, on cotton aphids and found that the most effective dose was 10^8 con/ml [98]. Santoro *et al.* (2008) experimented with various strains of *B. bassiana* on *Alphitobius diaperinus* Panzer (Coleoptera: Tenebrionidae) and they also stated that the most effective dose was $1x10^8$ con/ml [99]. Yücel *et al.* (2018) experimented 4 different doses ($1x10^5$, $1x10^6$, $1x10^7$, $1x10^8$ con/ml) of *B. bassiana* strain, which was isolated from mycosed larvae and adults of *H. postica* in Adana and Iğdır, on *Hypera postica* (Gyllenhall) (Coleoptera: Curculionidae) and found that its efective dose was $1x10^8$ con/ml [100]. These results were consistent with our findings. It was understood from these findings that the high concentration of the entomopathogenic fungus to be used as a biological control agent could give a better result. However, in biological control studies conducted by other researchers, it was stated that the high dose of different entomopathogenic species applied on harmful insects was low in virulence [101,102]. The virulence of the entomopathogenic fungus was stated by researchers that it not only depends on the strain of the fungus applied, but also on the dose, formulation and frequency of the dose applied [101].

As a result of the observations made during the experiments, LT_{50} values and growing time of spores on *Galleria* larvae were decreased due to the increase in concentration. Vu *et al.* (2008) stated that as conidial concentration increased, LT_{50} value decreased [103]. Luz *et al.* (1999) noted that entomopathogenic fungal spores applied to insects grow later and occur in smaller amounts on cadavers due to a decrease in concentration [77]. In experiments conducted by different researchers on the effectiveness of different doses of enomopathogen fungus strains on *G. mellonella* larvae, they emphasized that the higher concentration applied to larvae caused the lower LT_{50} value [104,105]. These findings were in line with our conclusions. It was understood from these findings that the virulence of different fungus species and different strains of the same fungus species could vary against the same insect pest.

So far as LD_{50} and LT_{50} results, it was found that the most effective strain was Erz strain and the weakest strain was MerK strains (Table 3-4). According to the results of the highest concentration application of all fungal strains, Erz and DenK1 strain were observed to kill 50% of *Galleria* larvae at the end of the second and third day (Table 4). As noted in many biological control application studies, this was

considered a successful outcome [106,107,108]. It was also observed that DenK1 strain was more effective at the highest and lowest doses than the standard strain (Table 4). The weakest strain at the lowest dose was the BarK2 strain (Table 4). However, statistical studies were shown that the isolated strains and the standard strains did not show a significant difference in their pathogenic effects against *G. mellonella* larvae or their potential as a biological control agent. This result was consistent with the results obtained by other researchers. In their study, they found that there was no significant difference between the mortality outcomes of different strains of *B. bassiana* tried on *G. mellonella* larvae [109]. But in another study, different isolates of each of the 4 species of entomopathogenic fungi, including *B. bassiana*, resulted in very different mortalities in the host organism being tried [57]. Ultimately, it was observed that these 10 *B. bassiana* strains attempted and the standard strain achieved successful mortality on *G. mellonella* larvae. According to statistical tests, the highest dose tried on larvae was found to provide the most successful control in each strain. This conclusion was paralleled with the results of studies conducted by other researchers [60,106,108,]. The effects of fungal strains were found not to be statistically significant, but overall the most successful strain was found to be Erz strain and the most unsuccessful strain was MerK strain.

However, these studies were carried out under laboratory conditions at a constant temperature, high humidity and dark conditions. These factors had positive or negative effects on the pathogenesis of entomopathogenic fungi, depending on the situation. Because pathogenicity depended not only on biochemistry, physiology, and molecular biology of disease development of the entomopathogenic fungus, but also on the environment. Relative humidity, UV rays, temperature and nutrient availability in these factors affected the performance of a successful mycoinsecticid [21,110]. Many researchers noted that UV rays render the conidias of entomopathogenic fungi inactive [110,111]. In addition, Wojda *et al*. (2009) stated that *G. mellonella* larvae were colony-dwelling insects, and when these larvae came together, the ambient temperature rised to 40 °C, thus providing protection against pathogens. This temperature had a negative effect on the development of fungal infection [112].

In later studies, it was aimed to look at how environmental conditions and insect behavior have an effect on the pathenities of *B. bassiana* strains that were tried on *Galleria* larvae. It was also thought that studying the effects of these strains on different pest insects could create useful data for subsequent biological control studies.

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