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# Natural infection potential and efficacy of the entomopathogenic fungus *Beauveria bassiana* against *Orosanga japonica* (Melichar)

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## Abstract

In this study, potential of the entomopathogenic fungi (EPF) isolates from dead samples of *Orosanga japonica* (Melichar) (Hemiptera: Ricaniidae) was evaluated. Infected specimens with fungi results confirmed that the samples were *Beauveria bassiana* after morphological and molecular identification. Amplicons produced two haplotypes ( $h = 2$ ), which included one polymorphic site after sequence and named isolates 1 and 2. Two haplotypes were rooted with MK229193.1, MG345084.1 *B. bassiana* Genbank samples. Two-year survey results of the EPF effects revealed the highest mortality and natural infection rates in the field. Natural mortality rates varied between 80.35% (Fındıklı-August) and 94.8% (Çayeli-September) in 2018 and between 79.82% (Alipaşa-August) and 97.75% (Fındıklı-September) in 2019. For nymphs, the lowest  $LT_{50}$  value was found at 2.92 days for isolate 1 and 2.56 days for isolate 2, with a concentration of  $1 \times 10^6$  conidia/ml, using the leaf dipping method. For adults, the lowest  $LT_{50}$  value was found at 3.02 days for isolate 1 and 3.15 days for isolate 2, with a concentration of  $1 \times 10^6$  conidia/ml, using the direct spraying method. Nymph  $LT_{50}$  values were found a little bit lower than adults. Direct spraying methods results gave a high  $LT_{50}$  value for nymph in contrast to adult. Although the  $LT_{50}$  ratios gave high/low degree in different isolates, methods and life stages, non-significant differences were found between each other's ( $p > 0.05$ ). In general, *B. bassiana* natural infection rates were found high in August and September during the 2 years. Efficacy of the two isolates, which derived from naturally infected *O. japonica* specimens, increased with concentration. The two isolates can potentially be used for *O. japonica* integrated management, as a fungal biocontrol agent, but their toxicological effects on beneficial insects, such as honeybees, will need to be determined.

**Keywords:** *Orosanga japonica*, *Beauveria bassiana*, Efficacy, Natural infection, Biocontrol

## Background

The Eastern Black Sea region in Turkey is characterized by a mild and rainy climate, favoring the survival of numerous insect pest species from tropical areas. This region has limited agricultural areas, and the most important agricultural products are tea, hazelnut, and kiwi. Insecticide usage is limited because of the

precipitation regime and the “zero chemical” perspective of the General Directorate of Tea Enterprises in Turkey.

*Orosanga japonica* (Melichar) (Hemiptera: Ricaniidae) is an invasive species in the western Palearctic region and widespread in the Black Sea area countries (Georgia, Ukraine, Russia, Turkey, and Iran) (Demir, 2018 and Mozaffarian, 2018). Rising population levels of *O. japonica* have become a considerable problem in the Eastern Black Sea region in Turkey, since the species was first detected in 2006 (Demir, 2009). *O. japonica* is a pest insect in tropical regions and recently, it was reported on

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chestnut in Abhazia, orchards in Iran as well as tea and kiwifruit in Turkey (Mozaffarian, 2018). Adults and nymphs feed on plant sap, and adults lay their eggs on a wide range of crop plants such as grape, kiwi, tea, beans, corn, cabbage, pepper, and eggplant (Gokturk and Aksu, 2014). Synthetic insecticides-based pest control is an important measure, but they also linked to adverse effects such as environmental contamination and insecticide resistance. Additionally, toxic and detrimental effects on non-target organisms are a problem in many areas (Dinesh et al. 2015). Although different types of biological control agents and mechanical control methods against the adult and nymph stages of *O. japonica* have been investigated (Ak et al. 2013 and Gokturk et al. 2018), more advanced studies are needed.

For insect control, the family Clavicipitaceae constitutes a favorable alternative in this regard (Sung et al. 2007), such as entomopathogenic fungi (EPF), which are distributed throughout many diverse ecological habitats (White et al. 2003). *Beauveria bassiana* is a well-known EPF species and has a worldwide distribution (Ownley et al. 2008). It belongs to the order Hypocrales (Ascomycota) and infects numerous insect species (Zimmermann, 2007). Several studies have investigated its effectiveness as a biological control agent in agricultural and forestry areas (Inanli et al. 2012 and Guven et al. 2015). *B. bassiana* can infect more than 70 insect pests, with a potential to control these species (Roy et al. 2006 and Van Lenteren, 2012).

The present study aimed to identify the EPF species that caused high rates of *O. japonica* mortality in the field, to determine natural infection rates under field conditions, and to confirm their efficacy against *O. japonica* under laboratory conditions.

## Material and methods

### Isolation of entomopathogenic fungi from naturally infected samples

Infected *O. japonica* specimens were collected from the field in 2018 around two locations in Rize city by hand picking. The specimens were placed in falcon tubes and transferred to the laboratory. Specimens collected on kiwifruit in Rize City Center and Findikli, whole collection points were fruit garden and situated around tea gardens area. Dead *O. japonica* specimens, infected with fungi, were transferred on potato dextrose agar (PDA) medium and incubated for 15–17 days at  $25 \pm 2$  °C in the dark and their spores were collected (Sevim et al. 2010) and transferred to falcon tubes containing 50 ml of Tween 80 solution (0.1% v/v). The conidia concentrations of the stock solution were determined via direct counting on a Thoma slide under a light microscope. Test conidia concentrations contained  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $1 \times 10^6$  conidia/ml for the bioassay. Each fungal set received a code (Rize

City Center isolate 1, Findikli isolate 2), and the collection area was noted (location, elevation, host plant, date). Isolates stored under sub-culture in PDA and sub-cultured every months according to the Oliveira et al. (2011) in the RTEU Vector Ecology and Control Laboratory in Rize Turkey.

### Determination of fungal isolates

EPF were identified morphologically and molecularly. Morphological species distinction was made according to Humber (1997). Morphologically identified species were confirmed molecularly for exact determination.

### DNA isolation, PCR, and analysis of the sequence data

The DNA isolation was performed, using the Gene JET genomic DNA Extraction Kit (Thermo Scientific®), according to the manufacturer's instructions. Isolated DNA amounts were measured Qbit and diluted for final PCR volume (50 µl). The ITS4-5 regions were amplified according to White et al. (1990). Each PCR reaction was performed, using T100™ Thermal Cycler–Bio-Rad®; the PCR mix contained 100 ng of genomic DNA, 1 x buffer, 2.5 mM MgCl<sub>2</sub>, 250 mM each dNTP, 100 nM each primer (ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATCG-3')) and 1 unit of Biolabs® Taq polymerase. Amplification conditions were as follows: 3 min at 95 °C for initial denaturation, 35 cycles of 60 s at 95 °C for denaturation, 30 s at an annealing temperature of 53 °C, 90 s at 72 °C for extension and 72 °C for 5 min for final extension. Amplicons were visualized using 1.5% agarose gel and sequenced at Macrogen Inc. (Amsterdam). The sequences were edited using the BIOEDIT program, version 7.0.5.2 (Hall, 1999) and aligned in CLUSTAL W (Thompson et al. 1994). The numbers of haplotypes (h) and polymorphic sites as well as nucleotide diversity ( $\pi$ ) were calculated using DNAsp version 5.0 (Rozas et al. 2017). The haplotypes identified in the present study were also compared to those available in GenBank samples. The neighbor-joining tree was generated by 1000 bootstrap replications according to the Mega 7 software (Kumar et al. 2016).

### Test organisms

Nymphs and adults of *O. japonica* were collected by an electric aspirator from four different localities at Rize in 2019 for the laboratory experiment. Specimens collected on kiwifruit in Rize City Center (Lon 41.0364 Lat 40.4934) and Findikli (Lon 41.2855 Lat 41.1715), on blackberry in Alipasa (Lon 41.0278 Lat 40.4494) and Cayeli (Lon 41.0963 Lat 40.7336). Whole collection points are fruit garden and situated around tea gardens area. Collection was performed with electric aspirator during 5 min and collected samples were placed in the

net cages. Collected samples were transferred to the laboratory and placed in previously sterilized rearing cages. Samples were kept at  $25 \pm 2$  °C,  $65 \pm 10\%$  RH and a photoperiod regime of 12:12 h light/dark in the insectarium. Fresh plant branches (blackberry) were placed into the cages for feeding. Fresh plant branches were washed by distilled water to remove soil and other pollutants, then washed with 85% ethyl alcohol solution and treated with distilled water to prevent infection of adults. Samples were kept for 120 h in the insectarium to acclimate to the environment and to avoid naturally infected specimens.

#### Survey of natural *Orosanga japonica* infections with *Beauveria bassiana*

In August and September 2018, wild *O. japonica* populations on host plants were surveyed for natural infection rates of *B. bassiana*. Surveys were performed monthly between August and September with an electric aspirator. The area had been checked monthly before the survey, but no infected samples were found. Counts were made over a period of 5 min by randomly selecting 3 trees (same species) infected and the *O. japonica* individuals were seen. Specimens were collected with and without visual sign with infection (dead and alive) during 5 min. Death/alive specimen ratio was evaluated as the sum of 3 plant collections. Collected death and alive individuals were placed in separate plastic boxes (34 × 22 × 12 cm) furnished with screen. Each individual was examined under a stereomicroscope for signs of infection by *B. bassiana*. Alive specimens were washed in a 0.5% sodium hypochlorite solution with 0.01% Triton X-100 after killing with ethyl acetate for avoiding any cuticular contamination. Then, they were dissected and rinsed twice with sterilized water then placed onto PDA and incubated at  $25 \pm 2$  °C for 2 weeks. The presence of fungal infection was evaluated visually. Infection rates were evaluated on all collected (dead, alive) samples.

#### Efficacy of *Beauveria bassiana* against *Orosanga japonica* in the laboratory

Laboratory assays were to evaluate the efficacy against *O. japonica* with 3 concentrations of *B. bassiana*. The conidia ratios for efficacy experiments were arranged at  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $1 \times 10^6$  conidia/ml in the suspensions (Tween 80 solution (0.1% v/v)). The direct effect of *B. bassiana* on nymphs and adults *O. japonica* was evaluated by leaf dipping and direct spray methods for leaves with slight modifications (Inanli et al. 2012 and Guven et al. 2015).

#### Leaf dipping bioassay

Field blackberry branches with leaves were collected and used. Collected 10 cm branches with leaves (4–5) were

selected. Branches were dipped in the conidia suspension (Tween 80 solution (0.1% v/v)) for 5 s and then kept at room temperature for 15–20 min for drying. A piece of cotton, moistened in 5 ml distilled water, was placed on the bottom of the glass jar. Branches with leaves were placed on top of the cotton to prevent drying. Then, 20 acclimatized individuals (nymphs and adults separately) were placed in the glass jars for testing. Tween 80 solution (0.1% v/v) was used as a control with the same procedure.

#### Direct spray bioassay

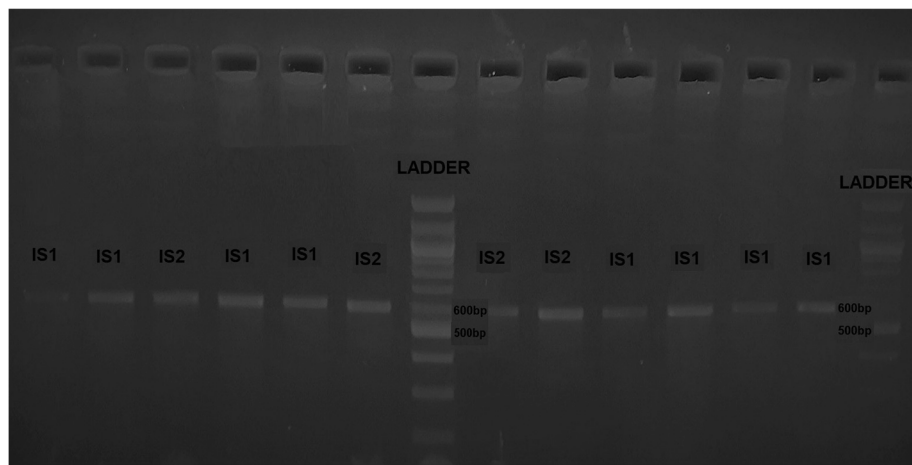
First, cotton moistened with 5 ml distilled water was placed on the bottom of the sterilized glass jar. Leaves were placed with the branches on top of the cotton to prevent drying. Then, 20 acclimatized individuals (nymphs and adults separately) were placed in the glass jars for testing. Sprays were performed with 1 ml of conidia suspension at a time in the glass jar (each concentration included the same amount of conidia) with hand sprayer. Sprayers washed with 1/10 diluted chromic acid and sterilized with 95% ethyl alcohol between tests. Tween 80 solution (0.1% v/v) was used as a control with the same procedure. The system was maintained at  $25 \pm 2$  °C, with a 12:12 light:dark cycle, over 9 days. Dead specimens were determined 3 times over those 9 days. Whole specimens were washed by sterilized water, placed onto potato dextrose agar (PDA) plates and incubated for 2 weeks at  $25 \pm 2$  °C in the dark. Colonies were determined microscopically. The presence of fungal infection was evaluated visually. Infection rates were evaluated on all (dead, alive) samples after testing.

#### Data analysis

Mortality rates were evaluated via Probit analysis ( $LT_{50}$ ). Differences between groups were analyzed via one-way ANOVA, and Tukey's test was applied for the evaluation between doses with  $p < 0.05$  significance level, using the IBM® SPSS® statistics program version 22.

#### Results and discussion

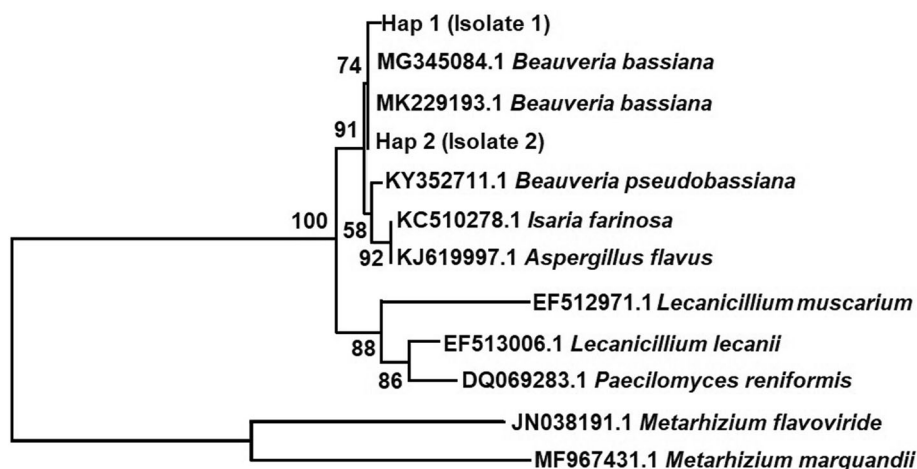
Two different fungal isolates were visually determined according to Humber (1997) as *B. bassiana*. The ITS4-5 region amplicons showed similar agarose gel electrophoresis images (Fig. 1). Sequence results were almost similar for 12 *B. bassiana* isolates. Amplicons produced 2 haplotypes ( $h = 2$ ), which included one polymorphic site; nucleotide diversity was  $\pi = 0.5333$ . Isolates gene sequences submitted to Genbank with MT350119 (RTEU1), MT350134 (RTEU2) Genbank accession number. The resulting tree had 2 distinct clades. Clade I encompassed JN038191.1 *Metarhizium flavoviridae* and MF967431.1 *M. marquandii* GenBank samples. The 2 haplotypes were rooted by MK229193.1, MG345084.1 *B.*



**Fig. 1** ITS4-5 amplicon gel electrophoresis results; IS1: isolate 1, IS2 isolate 2, 1, 5% gel electrophoresis, 45 min run with 60 V. 100-bp ladder was used for identification of the exact amount of amplicons

*bassiana* samples as a different branch in Clade II. Clade II also encompassed other GenBank fungal samples (*Lecanicillium muscarium* EF512971.1, *L. lecanii* EF513006.1, *Paecilomyces reniformis* DQ069283.1, *Isaria farinosa* KC510278.1, *Aspergillus flavus* KJ619997.1, *Beauveria pseudobassiana* KY352711.1) in Clade II (Fig. 2). Isolation and identification of the local pathogens are essential to possible manage pest species in an ecologically sustainable manner. Ribosomal RNA genes are conserved to different degrees and highly divergent spacer area. Internal transcribed spacer (ITS) sequence results have been widely used in fungal separation (Driver et al. 2000 and Cai et al. 2013). The two haplotypes were identified by one polymorphic site. Rehner and Buckley (2005) reported that the two unrelated and morphologically

indistinguishable clades from different *B. bassiana* isolates by using ITS1-5.8-ITS2 and EF-1a sequences. Sevim (2010) reported the 23.3% *B. bassiana* presence, which isolated from different soil resources in Eastern Black Sea area in Turkey and verified samples by using ITS1-5.8S-ITS2 region. This study reported that similarity 15 different isolates under one big clade together Rehner ve Buckley (2005) isolates. Ghikas et al. (2010) reported the two main clades for *B. bassiana* strains consisting *B. bassiana* (Clade A) and *B. pseudobassiana* (Clade C). NJ algorithm revealed that the isolates situated Clade A and separated *B. pseudobassiana* (Clade C) isolates with high bootstrap value. Although the result revealed the two different haplotypes with ITS region, it needs more detailed study for revealing phylogenetic relation with other isolates and



**Fig. 2** Phylogenetic relationships between haplotypes of *Beauveria bassiana* samples in the Eastern Black sea area and other entomopathogenic fungal species. The analysis was based on the neighbor-joining algorithm using the Tamura-Nei genetic distance model, with bootstrap support estimated from 1000 repetitions



genetic variation by different genetic markers. Even though many authors reported the *B. bassiana* populations have low genetic diversity (Gaitan et al. 2002), *Beauveria* isolates showed differences for the virulence (Cruz et al. 2006). Some studies also reported that the genetic homogeneity for *B. bassiana* isolates from a small geographical regions/areas (Muro et al. 2005 and Fernandes et al. 2006). Mar et al. (2012) reported that 99% similarity between the two *B. bassiana* isolates from different sites and hosts in Northern Thailand. Obtained *B. bassiana* isolates originating from different points but from the same host. Similarly, the sequences showed a high similarity and one mutational point.

#### Natural infection of *Orosanga japonica* with *Beauveria bassiana*

The insect species *O. japonica* is widely distributed in the Eastern Black Sea area and has also been found in the western part of the Black Sea (Demir, 2018 and Arslangundogdu and Hizal 2019). Obtained results also showed that from 2018 to 2019, the population of this species increased. In the 2018 and 2019 active seasons, *O. japonica* adult specimens, naturally infected with *B. bassiana*, were collected from 4 different locations in Rize City (Table 1). Big epizootic situation has not detected yet. Naturally infected specimens are shown in Fig. 3.

In 2018, 2325 specimens were collected during the field study with 5 min/human collection from 4 different collection points. Mortality rates (NDS%) ranged between 80.35% (Fındıklı-August) and 94.8% (Çayeli-September). Actual infection rates (AIS%) showed similarity and were higher than mortality rates. Mortality and actual infection rates were higher in September than in August. In August, the mortality rate in Rize City (88.64%) was higher than in other areas. The lowest

mortality rate was found in Fındıklı (80.35%). In September 2018, the highest mortality rate was found in Çayeli (94.8). A correlation between mortality rates and infected specimen number was observed.

In 2019, 3192 specimens were collected. Mortality rates ranged between 79.82% (Alipaşa- August) and 97.75% (Fındıklı-September). In August, Fındıklı showed the highest mortality rate (97.4%). The lowest mortality rate (79.82%) and actual infection rate (81.35%) were found in Alipaşa. In September, the highest mortality rate was found for Fındıklı (97.75%), similar to August, with a correlation with the actual infection rate. The lowest mortality rate was found for Rize City (95.62%) and the lowest actual infection rate for Çayeli (98.54%). In general, more specimens were collected in August than in September, although the mortality and actual infection rates were higher in September than in August. In 2019, more specimens were collected than in 2018 (Table 1).

*B. bassiana* occurred in various natural soils and is not related to a certain region (Bing and Xing 2008). This species has been isolated from several insect hosts in nature from different regions, including Turkey (Padmavathi et al. 2003).

#### Efficacy of *B. bassiana* against *O. japonica* in the laboratory

The results of the efficacy of the 2 different isolates of *B. bassiana* with the 2 different application methods against *O. japonica* nymphs are presented in Table 2. For isolate 1, leaf dipping method  $LT_{50}$  results varied between 6.634 ( $1 \times 10^4$ ) and 2.92 days ( $1 \times 10^6$ ). Actual infection results showed correlation and were slightly higher than the mortality rates. For isolate 2, leaf dipping method  $LT_{50}$  results varied between 7.23 ( $1 \times 10^4$ ) and 2.56 days ( $1 \times 10^6$ ). The most efficient results were found via applying the leaf dipping method at the concentration of  $1 \times 10^6$  conidia/ml concentration, and the  $LT_{50}$  time decreased to almost 2.56 days for isolate 2 at a  $1 \times 10^6$  conidia/ml concentration.

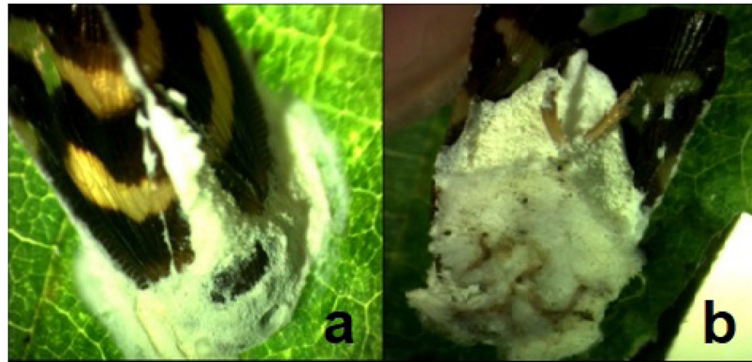
The results regarding the efficacy of the two different isolates of *B. bassiana* with two different methods against *O. japonica* adults are presented in Table 3. Similarity with the nymphs' results and consistency between the 2 isolates were noticed. The most efficient results were found via direct spraying at a concentration of  $1 \times 10^6$  conidia/ml, and the  $LT_{50}$  time decreased to almost 3.02 days for isolate 1 at a concentration of  $1 \times 10^6$  conidia/ml.

In general, direct spraying mortality showed a higher degree than the leaf dipping method, but without a significant difference ( $p > 0.05$ ) ( $F = 0.79$ , significance = 0.37). The application of isolate 2 resulted in a slightly higher  $LT_{50}$  value than isolate 1 for the same concentration ( $p > 0.05$ ) ( $F = 0.09$  significance = 0.762). Although adult

**Table 1** Mortality rate and number of specimens infected with *Beauveria bassiana* over 2018 and 2019

Area	August 2018					September 2018				
	N	NDS	NDS%	AIS	AIS%	N	NDS	NDS%	AIS	AIS%
Alipaşa	302	246	81.45	254	84.1	225	194	86.22	207	92
Rize	458	406	88.64	411	89.73	199	176	88.44	189	94.97
Çayeli	382	321	84.03	337	88.21	250	237	94.8	242	96.8
Fındıklı	280	225	80.35	262	93.57	229	208	90.82	211	92.13
Area	August 2019					September 2019				
	N	NDS	NDS%	AIS	AIS%	N	NDS	NDS%	AIS	AIS%
Alipaşa	456	364	79.82	371	81.35	210	205	97.61	208	99.04
Rize	583	501	85.93	519	89.02	343	328	95.62	341	99.41
Çayeli	489	461	94.27	462	94.47	412	397	96.35	406	98.54
Fındıklı	386	376	97.4	380	98.44	356	348	97.75	355	99.71

Note: N number of specimens collected during 5 min. NDS number of dead specimens, AIS actual infected specimens



**Fig. 3** Adult specimens of *Orosanga japonica* infected with *Beauveria bassiana* in the field. **a** Infected with isolate 2. **b** Infected with isolate 1

LT<sub>50</sub> results were generally higher than those for nymphs, non-significant differences ( $p > 0.05$ ) ( $F = 0.196$ , significance = 0.66) between nymphs and adults were found. The ANOVA results for concentration effects showed significant differences between concentrations ( $F = 0.196$  significance = 0.000). Tukey's test results showed differences ( $p < 0.05$ ) between the first ( $1 \times 10^4$  conidia/ml) and the other two concentrations (significance between 1st and 2nd dose = 0.02; significance between first ( $1 \times 10^4$  conidia/ml) and third ( $1 \times 10^6$  conidia/ml) concentration = 0.000). The second ( $1 \times 10^5$  conidia/ml) and third ( $1 \times 10^6$  conidia/ml) concentrations were non-significantly different (significance = 0.08), but close to the  $p < 0.05$  level.

Three different concentrations against nymphs and adults were tested, of which the concentration of  $1 \times 10^6$

conidia/ml was the most effective. The highest efficacy of local *B. bassiana* isolates against two life stages of *O. japonica* was reported. The LT<sub>50</sub> values decreased with increasing dosage. Numerous studies have investigated the effectiveness of EPF in biological pest control, but studies using *O. japonica* are limited (Ak et al. 2013 and Gokturk et al. 2018). Guclu et al. (2010) reported that nymphs were more sensitive than adults against the EPF *Lecanicillium muscarium*, and *Lecanicillium muscarium*. The LT<sub>50</sub> values ranged from 2.34 to 3.90 days, with a single concentration ( $1 \times 10^7$  conidia/ml) against nymphs. Ak et al. (2014) also reported that the *Conidobolus coronatus* strain Cc3 had potential in the *O. japonica* IPM program. LT<sub>50</sub> value variation was from 3.19 to 3.66 days with three different *C. coronatus* isolates in a

**Table 2** Leaf dipping and direct spraying LT<sub>50</sub> values against *Orosanga japonica* nymphs

	Isolate 1					Isolate 2				
	Dose (conidia/ml)	Mortality/actual infection %	LT <sub>50</sub> (days)	Lower bound	Upper bound	Mortality/actual infection %	LT <sub>50</sub> (days)	Lower bound	Upper bound	
Leaf dipping	$1 \times 10^4$	74	6.634	ND	ND	72	7.23	ND	ND	
	$1 \times 10^5$	78	4.721	4.145	5.282	79	5.68	ND	ND	
	$1 \times 10^6$	89	2.922	2.159	3.505	88	2.563	0.702	3.614	
	Control	2		ND	ND	4				
Direct spraying	$1 \times 10^4$	71	5.859	ND	ND	70	6.958	ND	ND	
	$1 \times 10^5$	90	3.947	ND	ND	88	4.419	3.58	5.07	
	$1 \times 10^6$	96	3.026	ND	ND	90	2.854	1.328	3.778	
	Control	3				5				
Leaf dipping actual infection	$1 \times 10^4$	76				77				
	$1 \times 10^5$	83				81				
	$1 \times 10^6$	94				95				
	Control	2				3				
Direct spraying actual infection	$1 \times 10^4$	80				81				
	$1 \times 10^5$	96				94				
	$1 \times 10^6$	95				93				
	Control	3				4				

**Table 3** Leaf dipping and direct spraying LT<sub>50</sub> values against *Orosanga japonica* adults

	Isolate 1					Isolate 2				
	Dose (conidia/ml)	Mortality/actual infection %	LT <sub>50</sub>	Lower bound	Upper bound	Mortality/actual infection %	LT <sub>50</sub>	Lower bound	Upper bound	
Leaf dipping	1 × 10 <sup>4</sup>	67	7.416	ND	ND	65	7.715	7.146	8.427	
	1 × 10 <sup>5</sup>	83	5.027	ND	ND	80	5.652	5.021	6.248	
	1 × 10 <sup>6</sup>	94	3.541	3.014	3.992	89	3.675	2.585	4.426	
	Control	7				5				
Direct spraying	1 × 10 <sup>4</sup>	71	6.618	ND	ND	70	7.061	ND	ND	
	1 × 10 <sup>5</sup>	88	4.301	ND	ND	88	4.896	4.236	5.461	
	1 × 10 <sup>6</sup>	96	3.026	ND	ND	93	3.153	2.002	3.917	
	Control	3				6				
Leaf dipping actual infection	1 × 10 <sup>4</sup>	70				72				
	1 × 10 <sup>5</sup>	90				88				
	1 × 10 <sup>6</sup>	98				95				
	Control	3				6				
Direct spraying actual infection	1 × 10 <sup>4</sup>	79				81				
	1 × 10 <sup>5</sup>	93				95				
	1 × 10 <sup>6</sup>	99				94				
	Control	2				3				

fixed 1 × 10<sup>7</sup> conidia/ml concentration against nymphs. Obtained results varied from 2.92 to 3.026 days with *B. bassiana* against nymphs and found that the best concentration was 1 × 10<sup>6</sup> conidia/ml. This variation and concentration efficiency may explain with EPF species and their virulence. Eken et al. (2006) reported that LT<sub>50</sub> value variation from 4.6 to 4.4 days with 1 × 10<sup>7</sup> and 1 × 10<sup>8</sup> conidia/ml for *B. bassiana* against *Saperda populnea*. Tuncer et al. (2018) showed that the 4.64 and 8.53 days LT<sub>50</sub> value against *Curculio nucum* adults with one *B. Bassiana* isolate and commercially product in a fixed 1 × 10<sup>8</sup> conidia/ml concentration. Obtained results showed the variations around the 3–8 days LT<sub>50</sub> values testing 3 different concentrations. These results implied that the from 1 × 10<sup>6</sup> to 1 × 10<sup>8</sup> concentrations were the best scale for the possible usage of *B. bassiana* isolates as a bio control agent. Göktürk et al. (2018) reported bio-pesticide efficiency against *O. japonica* and found a low efficacy for *B. bassiana* against nymphs and adults (under 20%). In contrast, obtained results showed a high efficacy against *O. japonica* nymphs and adults with the two natural *B. bassiana* isolates and a high rate of natural infection. These differences may explain preserving conditions after isolation from the field or usage of different test method. Our isolates preserved sub-culturing condition according to the Oliveira et al. (2011) who reported that the sub-culturing was the best method for *B. bassiana* preserving. Gokturk et al. (2018) preserved the isolated samples at – 86 °C before the identification and did not give information about the keep condition before and after tests.

Several EPF showed different degrees of efficacy against this pest species, but further detailed studies are necessary to develop environmentally friendly and economically viable biocontrol options. The species *B. bassiana* may be a good control agent for this pest species, but area conditions and toxicity against other arthropods are important when considering commercially usage of it. The Eastern Black Sea area is an important honeybee production area for Turkey and has economically important honey products. There are some reports of the negative effects of *B. bassiana* on the honeybee nest mate recognition ability and longevity (Potrich et al. 2018 and Cappa et al. 2019). The EFSA (European Food Safety Authority) has also released a review of the effects of *B. bassiana* on different animal, soil, and plant species, including the bee brood and nest mate recognition (EFSA, 2015). Against this context, more detailed studies on different isolates, persistence, and toxicological effects on non-target organisms are needed.

### Conclusion

EPFs have become an important component for pest control. *B. bassiana* is one of the most studied and well-known EPFs. Obtained results revealed positive potentials against the nymphs and adults of *O. japonica* under field and laboratory conditions. *B. bassiana* could be a good component for the area wide insect pest management options for *O. japonica* but further detailed studies related to the non-target organisms' effects are still needed.

**Abbreviations**

ANOVA: Analysis of variance; *ITS*: Internal transcribed spacer; *h*: Haplotype;  $\pi$ : Nucleotide diversity; LT<sub>50</sub>: Lethal time of the 50% of the population died; NDS: Number of dead specimens; AIS: Actual infected specimens; *N*: Number of specimens collected during 5 min; EFSA: European Food Safety Authority; EFP: Entomopathogenic fungus

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**Authors' contributions**

MMA, AU, and MO designed the experiment. MO, IG, and AU conducted the experiment and wrote the article. MO performed statistical analysis. MMA and AU revised the article. All authors approved the final article after reading.

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**Availability of data and materials**

All the data in this study have been presented in this manuscript except laboratory bioassay data for LT<sub>50</sub>. The analyzed data for LT<sub>50</sub> are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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