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Development of mycoinsecticide formulations with *Beauveria bassiana* and *Metarhizium brunneum* for the control of *Orosanga japonica* (Hemiptera: Ricaniidae)

Seda Biryol[1] | Ebru Güney[1] | Ardahan Eski[2,3] | Zeynep Bayramoğlu[4] | Kazım Sezen[1] | Zihni Demirbag[1] | İsmail Demir[1]

[1]Department of Biology, Faculty of Science, Karadeniz Technical University, Trabzon, Turkey

[2]Program of Biomedical Equipment Technology, Bilecik Şeyh Edebali University, Vocational School, Bilecik, Turkey

[3]Biotechnology Application and Research Centre, Bilecik Şeyh Edebali University, Bilecik, Turkey

[4]Department of Plant and Animal Production, Recep Tayyip Erdoğan University, Pazar Vocational School, Pazar, Rize, Turkey

Correspondence
İsmail Demir, Department of Biology, Faculty of Science, Karadeniz Technical University, 61080 Trabzon, Turkey.
Email: idemir@ktu.edu.tr

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Abstract

Entomopathogenic fungi are promising microbial agents for agricultural pests, especially piercing-sucking insects. In this study, eight indigenous fungal isolates including four isolates of *Metarhizium brunneum* and four isolates of *Beauveria bassiana* were tested on *Orosanga japonica* Melichar (Hemiptera: Ricaniidae) in order to find an effective control agent that can be utilised for the development of oil-based fungal mycoinsecticides. In the screening test using 1×10^7 spores ml^{-1} concentration, KTU-24 (*B. bassiana*) and KTU-51 (*M. brunneum*) had the highest lethal activity against nymphs and adults of the target pest. Also, at the highest concentration (10^9 spores ml^{-1}), KTU-24 showed 92.33 and 94.88% mortality on nymphs and adults at the dose–response tests, respectively, and the KTU-51 isolate showed 100% mortality on both stages of *O. japonica* under laboratory conditions. With these results, KTU-24 and KTU-51 isolates were used to develop biopesticides. While the mass production of *B. bassiana* was carried out with liquid-state fermentation, solid-state fermentation was used for mass production of *M. brunneum*. Spores of both isolates were formulated in oil, and products were named as RICANICIDAL *Bbas*-TR61 and RICANICIDAL *Met*-TR61, respectively. Formulations caused over 97% mortality against nymphs and adults of *O. japonica* under field conditions and both formulations showed higher efficacy than commercial mycoinsecticides at 10^8 spores/ml concentration 20 days after treatment. The currents study shows that both mycoinsecticide formulations are highly promising for management of *O. japonica*.

KEYWORDS
Beauveria, biological control, *Metarhizium*, oil-based mycoinsecticide, *Orosanga japonica*

1 | INTRODUCTION

Orosanga japonica Melichar (syn. *Ricania japonica*) (Hemiptera: Ricaniidae) is an invasive and polyphagous insect in Turkey that can be found on trees, shrubs and weeds. Nymphs and adults of the pest cause serious damage by feeding on various vegetables and fruits, including cucumber (*Cucumis sativus* L.), fig (*Ficus carica* L.), grapevine (*Vitis vinifera* L.), cabbage (*Brassica oleracea* L.), kiwi (*Actinidia chinensis* Planch.), citrus fruits (*Citrus sinensis* L.), corn (*Zea mays* L.), beans (*Phaseolus vulgaris* L.), mulberry (*Morus alba* L.), hazelnut (*Corylus avellana* L.) and tea (*Camellia sinensis* L.) (Demir, 2009, 2018; Oztemir, 2014; Cebir, 2016; Akiner et al., 2019). *O. japonica* is native to Far East Asia, including China, Japan and Korea and has spread to Georgia, Crimea, Krasnodar and Iran (EPPO, 2016; Gnezdilov & Sugonyaev, 2009; Nast, 1987). In Turkey was reported in 2007, around the vicinity of Rize (Eastern Black Sea Region) (Demir, 2009). However, *O. japonica* was misidentified as *Ricania simulans*

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(Walker) (EPPO, 2016). Studies on biological control of this pest classified as *R. simulans* have been conducted (Ak, Güçlü, & Sekban, 2013; Gokturk & Aksu 2014; Gokturk & Mıhlı, 2015; Güçlü et al., 2010). However, the controversial topic was clarified by Akiner et al. (2019) and the insects collected in the Eastern Black Sea Region of Turkey were found to be *O. japonica* according to the detailed morphological examinations and molecular characterisation.

Turkey is one of the important tea producer countries in the world. Regarding world tea production in the world, Turkey ranks in fifth place after China, India, Kenya and Sri Lanka (Yurteri, Ozcan, & Seyis, 2019). In Turkey, tea cultivation areas are only found in the Eastern Black Sea Region and almost all areas were infested by *O. japonica*. Although the most convenient method to control this pest is the use of chemical insecticides, it is prohibited by General Directorate of Tea Enterprises (Yurteri et al., 2019). For this reason, mechanical control practices have been recommended for this pest, including egg destruction before hatch (Altas & Ak, 2019). In spite of these management practices, successful control of *O. japonica* has been difficult due to its rapid development and reproduction rate. Therefore, population densities have increased rapidly in the years following its introduction, and has made it difficult for producers to manage (Cebir, 2016; Oztemir, 2014). Given these difficulties with managing the pest, more effective and safe alternative strategies are urgently needed.

These alternative methods of control include the use of the entomopathogenic fungi *Lecanicillium muscarium* (Güçlü et al., 2010), *Conidiobolus coronatus* (Ak, Eken, Güçlü, Genç, & Sekban, 2014), azadirachtin and spinosad based biopesticides (Ak et al., 2013), Pyrethum, *Bacillus thuringiensis* (Gokturk and Mıhlı (2015)), culturable endosymbiotic bacteria such as *Pseudomonas* sp., *B. safensis* and *B. thuringiensis* (Alev & Sezen, 2016), and light traps (Gokturk & Mıhlı, 2016). Gokturk, Kordalı, and Bozhüyük (2017) indicated that the essential oils of *Rosmarinus officinalis* killed 47.5 and 33% of nymphs and adults, respectively. In another study, Gokturk, Tozlu, and Kotan (2018) noted that *Beauveria bassiana* isolate and *B. thuringiensis* subsp. *kenyae* both had 42% insecticidal activity.

The studies mentioned above were generally carried out under the laboratory; yet no effective biocontrol method has been applied in the field conditions. In addition, development of mycoinsecticide from an indigenous fungus has not been performed until now. Therefore, this study was carried out under laboratory and field (cages) condition to evaluate the feasibility of developed oil formulations based on *B. bassiana* and *Metarhizium brunneum* to control *O. japonica* in caged tea plants.

2 | MATERIALS AND METHODS

2.1 | Origin of the fungi

The fungi (four × *Metarhizum* and four × *Beauveria*) used in the current study were obtained from the entomopathogenic culture collection of Microbiology Laboratory, Department of Biology, Karadeniz Technical University. In earlier studies, they have been isolated from soil and insect samples in the Eastern Black Sea Region and they showed significant insecticidal effect on various pests (Table 1) (Sevim, Demir, & Demirbag, 2010; Sevim, Demir, Hofte, et al., 2010; Sevim, Demir, Tanyeli, & Demirbag, 2010; Yucel et al., 2018)

2.2 | Preparation of spore suspension

A loopful of fungal spores from stock culture were transferred on potato dextrose agar (PDA) plates and incubated for 3 days at 25 ± 2°C under total darkness. After incubation, a single spore was transferred to new PDA medium and incubated for 2 weeks until plates were fully overgrown. Then, fungal spores were harvested from the plates using a sterile scalpel and transferred to 10 ml of sterile 0.01% Tween 80. The conidial suspensions were filtered with sterile muslin to remove hyphal debris and vortexed for 2 min to become homogeneous. The concentration of spores in the final suspension was determined using a Neubauer haemocytometer.

2.3 | Screening tests

Screening tests were performed at a concentration of 10^7 conidia ml^{-1} against the third instar nymphs and adults of *O. japonica*, separately. Insects were collected from infested fields and fed with fresh tea plants for a week to adapt to laboratory conditions. Experiments were carried out with 50 nymphs and 50 adults for each fungus, included three replicates, and were repeated three times on separate days. Insects were put into disinfected plastic boxes (15 cm × 20 cm) containing tea shoots stuck in water agar, and 1 ml conidial suspension was applied by a mini hand-sprayer (10 ml). Sterile Tween 80 (0.01% vol:vol) was used in control group (Sigma-Aldrich P1754, Darmstadt, Germany). Insects were held in environmental chambers at 25°C, 60% RH and 12:12 (L:D). Insects were counted 10 days after application, and mortality rates were calculated. The cadavers were surface sterilised with 0.2% sodium hypochlorite solution for 3 min, rinsed with sterile distilled water and assessed for mycosis by placing them in a moisture chamber to stimulate fungal sporulation.

2.4 | Dose response experiments

B. bassiana (KTU-24) and *M. brunneum* (KTU-51), which had the highest insecticidal effects in the screening tests, were used in the dose–response experiments. Conidial suspensions from both species were serially diluted in 0.01% Tween 80 and, six different concentrations from 10^9 to 10^5 conidia ml^{-1}, were applied to third instar nymphs and adults of *O. japonica*. Experiments were conducted as indicated in the screening test by Kocacevik, Sevim, Eroglu, Demirbag, and Demir (2015).

2.5 | Cage field trial

The efficacy of the fungi (KTU-24 and KTU-51) used in the dose–response experiments were tested against both nymphs and adults of

TABLE 1 Entomopathogen fungi used in bioassay study

Isolate code	Source	Species	Reference
KTU-24 (ÇK)	*Thaumetopea pityocampa*	*Beauveria bassiana*	Sevim, Demir, and Demirbag (2010)
KTU-57 (Rh)	*Rhynchites bacchus*	*B. bassiana*	Sevim, Demir, Tanyeli, and Demirbag (2010)
K4	*Hypera postica*	*B. bassiana*	Yucel, Gozuacik, Gencer, Demir, and Demirbag (2018)
Pa4	*Pristiphora abietina*	*B. bassiana*	Biryol et al. (2021)
KTU-2 (Ardeşen)	Soil	*Metarhizium anisopliae*	Sevim, Demir, Tanyeli, and Demirbag (2010)
KTU-40 (53)	Soil	*M. anisopliae*	Sevim, Demir, Tanyeli, and Demirbag (2010)
KTU-51 (4-Güm-A)	Soil	*M. anisopliae*	Sevim, Demir, Tanyeli, and Demirbag (2010)
KTU-21 (117)	Soil	*M. anisopliae*	Sevim, Demir, Tanyeli, and Demirbag (2010)

O. japonica in cage under field conditions. Insects were collected from the infested tea planting area and, third instar nymphs ($n = 50$) and adults ($n = 50$) were separately placed in tulle cages (25 cm × 35 cm). Total volume of 5 ml of conidial suspension was sprayed to each cage at 10^7 conidia ml^{-1} concentration. In the control treatment, same amount of sterile Tween 80 (0.01% vol:vol) was sprayed. Each tulle cage was wrapped to two to three tea shoots and tightly bound to prevent insects escaping. Experiment was repeated five times for nymphs and adults, and mortality rates were evaluated 20 days after application.

2.6 | Effects of temperature and ultraviolet on radial growth and sporulation

The experiments were perfumed according to the method of Braga, Flint, Messias, Anderson, and Roberts (2001) with some modifications. Fungal spores were obtained from 2-week-old cultures with the same methods described in Section 2.2, and concentration of spores was determined using a Neubauer haemocytometer. Spores of KTU-24 and KTU51 (10 µl, 10^4 conidia ml^{-1}) were transferred onto Saboraud dextrose yeast extract agar (SDAY) medium supplemented with chloramphenicol (50 mg ml^{-1}) and incubated at 20, 28, 30 and 37°C. For ultraviolet (UV) experiments, Petri dishes were exposed to 306 nm wavelength UV light for 30 and 60 min after fungal spore inoculation. Control groups were not exposed to UV radiation. Then, the plates were incubated in the dark for 7 days and radial mycelial growth was measured using an inverted microscope (Ali-Shtayeh, Abdel-Basit, & Jamous, 2003). Then, plates were incubated for one more week and assessed for spore production. To determine the effect of temperature and UV on sporulation, plates were incubated for 14 days and spore harvesting, and determination of concentration were performed as described above. Experiments were replicated three times on separate days.

2.7 | Blastospore production of *B. bassiana* KTU-24 in fermenter

Before switching from flask to the fermenter, *B. bassiana* KTU-24 isolate was produced in a small volume of basal salt medium as an initial culture (Jackson, McGuire, Lacey, & Wraight, 1997). After this period, 10 ml sterile 0.1% Tween 80 was added to the Petri dish, and spores were obtained by scraping with Drigalski spatula. The spore suspension was counted with a Neubauer haemocytometer, and the concentration was adjusted to 10^6 conidia ml^{-1}. Then, 15 ml of this suspension was inoculated into 150 ml basal salt medium as an initial culture in 250 ml flasks and left to incubate for 3 days at 25°C at 350 rpm (Mascarin, Jackson, Kobori, Behle, & Delalibera, 2015; Mascarin, Jackson, Kobori, Behle, Dunlap, & Delalibera, 2015). The fermenter, including for every 1,000 ml basal salt medium, was inoculated with 10 ml initial culture containing 10^6 blastospores ml^{-1}. Fermentation was carried out for 3 days at 350 rpm, 25°C and pH 5.3 (Mascarin, Jackson, Kobori, Behle, & Delalibera, 2015; Mascarin, Jackson, Kobori, Behle, Dunlap, & Delalibera, 2015). After the incubation, the blastospores were filtered through a sieve [200 mm × 50 mm stainless steel test sieve, 710 µm (No: 25) (Retsch, Germany)], and the hyphae structures were removed by centrifugation at 10,000*g* for 10 min. Blastospores were suspended in sterile water, and the concentration was adjusted to a minimum of 2×10^{11} conidia ml^{-1}.

The blastospores produced in the fermenter were mixed with skim milk powder because a composite encapsulation matrix was prepared by skim milk in a 10% (wt/vol) ratio. The mixture was spray dried by a laboratory scale spray dryer equipped with two-fluid nozzle (SD-Basic, Lab Plant, UK). The drying process was performed by adjusting internal temperature to 95 ± 2°C, and outdoor temperature 48 ± 2°C, feedback rate was performed in 20 ml min^{-1}, 5.7–5.9 bar air pressure (Mascarin et al., 2015). The dried spores were stored in +4°C until used in oil formulations.

2.8 | Conidiospore production of *M. brunneum* KTU-51 in solid substrate

Before switching to solid substrate, *M. brunneum* KTU-51 isolate must be produced in liquid medium. Therefore, the isolate was cultured with the same methods described in Section 2.2. The spore suspension was counted with a Neubauer haemocytometer and the concentration was adjusted to 2.9×10^6 conidia ml^{-1}. The liquid medium (1 L; 30 g glucose or sucrose, 20 g yeast extract, 4 g potassium hydrogen phosphate, 25 g casein hydrolyzate, 10 mg gentamicin and

pH 5.6) was inoculated with 100 ml of fungal suspension, and incubated at 28°C at 150 rpm for 4 days (Seema, Neeraj, & Krishan, 2013). Conidiospores were centrifuged at 10,000 rpm for 1 min, and the collected spores were dissolved in sterile distilled water. Concentration was adjusted to 5×10^8 conidia ml^{-1}.

Rice was used for solid-state fermentation. Rice was soaked in 70°C water for 60 min, and the excess water was drained completely. Then, 150 g of semi-cooked rice was packed in a polypropylene bag and autoclaved at 121°C at 1.1 atm pressure for 40 min (Seema et al., 2013). After cooling, 7.5 ml of conidial suspension was inoculated into the autoclaved bags under laminar air flow chamber. The bags were shaken for homogeneous distribution of fungal spores in the medium. Then, bags were kept in incubator at 25°C for 20 days and shaken every 5 days to avoid clumping and providing more surface area for fungal growth during incubation.

The rice coated with fungal bodies was transferred to Kraft paper bags (15 cm × 30 cm) and allowed to pre-dry for 10 days. Then, fungal spores were harvested by using a sieve (45 μm $mesh^{-1}$), and were dried in a vacuum desiccator until the humidity was less than 5%. The fungal spores were individually packaged in 100 ml glass bottles and stored in a refrigerator with low humidity capacity at 4°C until they were used in oil formulations.

2.9 | Development of mycoinsecticides

Oil formulations for the fungi were developed to extend their shelf life before applications. Spore powder of *B. bassiana* KTU-24 and *M. brunneum* KTU-51 were used to develop mycoinsecticides. The oil formulation developed by Nian, He, Lu, and Zhao (2015) was used with some modifications. The formulations were prepared by mixing naphthalene-2-sulfonic acid (3%), lecithin (6%), silvet L-77 (1%), ascorbic acid (0.1%), sodium alginate (1%) and vegetable oil (73.9%) with 10 g spore powder (10^{10} spore g^{-1}). The mixture was completed to 100 ml with sterile distilled water and stirred gently (50 rpm) for 5 min below 10°C until homogenous (approximately 30 min). The spore count of the formulation was performed using serial dilutions and a Neubauer haemocytometer.

2.10 | Efficacy of mycoinsecticides against *O. japonica*

The KTU-24 and KTU-51 formulations and two commercial products, Met52 (*Metarhizium anisopliae* strain F52, 9×10^8 conidia/ml) and Nostalgist BL (*B. bassiana* strain Bb-1, 1×10^8 conidia ml^{-1}) were tested against third instar nymphs and adults of *O. japonica* at the concentration of 10^8 conidia ml^{-1} under laboratory conditions using the method described in the screening test. Then, our formulations and the two commercial products with the concentration of 10^8 conidia ml^{-1} were tested on caged first and third instar nymphs and adults of *O. japonica* under field conditions. Fungus-free oil formulation and sterile distilled water were used as negative control. Only KTU-24 and KTU- 51 formulations were tested on first and third instar nymphs, and adults of the pest under field conditions using three different concentrations (from 10^9 to 10^7). Field experiments were repeated five times.

2.11 | Statistical analysis

The mortality rates were calculated and corrected according to Abbott formula (Abbott, 1925). The data were subjected to one-way analysis of variance followed by least significant difference tests for post hoc comparison of means. Concentration mortality data were subjected to probit regression analysis and median lethal concentration (LC_{50}) was estimated (Finney, 1971). All analyses were performed using SPSS 25.0 statistical software (IBM, Armonk, NY).

3 | RESULTS

The fungal isolates used for the screening tests, caused mortality between 58 and 100% on nymphs, and also between 57 and 98% on the adults. Amongst these isolates, *B. bassiana* KTU-24 and *M. brunneum* KTU-51 isolates showed the highest insecticidal activities against *O. japonica*. While KTU-24 isolate caused 100 and 98% mortality against the nymphs and the adults, KTU-51 isolate yielded 95 and 90% mortality on nymphs and the adults, respectively (Figure 1). The emerging of whitish and greenish conidiospores from the cadavers indicated that the mortalities were caused by the fungal isolates.

The dose–response experiments were performed with two isolates (KTU-24 and KTU-51) which had the highest insecticidal effect at laboratory conditions. KTU-24 showed a rapid effect on the pest; however, no significant difference was observed between KTU-24 and KTU-51 mortality rates at the end of the application. It was determined that the effectiveness of both isolates on the nymphs and adults of pest increased by increasing the doses. KTU-24 caused 92.33 and 94.88% mortalities with 10^9 conidia ml^{-1} concentration on the nymphs and the adults at 10 days after treatment, respectively. On the other hand, 100% mortality was determined with KTU-51 on both stages of the pest with the same concentration and at the same conditions (Figure 2).

Also, median lethal concentrations (LC_{50}) on the third instar nymphs were determined as 6.08×10^5 and 1.7×10^5 conidia ml^{-1} for KTU-24 and KTU-51, respectively. However, LC_{50} values of the isolates on adults could not be calculated because they had a very high mortality at the tested concentrations (Table 2).

Since these results should be supported by the field trials, efficiency of the fungal isolates were evaluated at the concentration of 10^7 conidia ml^{-1} under the semi-field conditions. Mortality rate on nymphs and adults reached over 80% at 20 days after application. It was determined that while KTU-24 was more effective against the nymphs with 85% mortality, KTU-51 was more effective against adults with 90% mortality. However, there are no significant

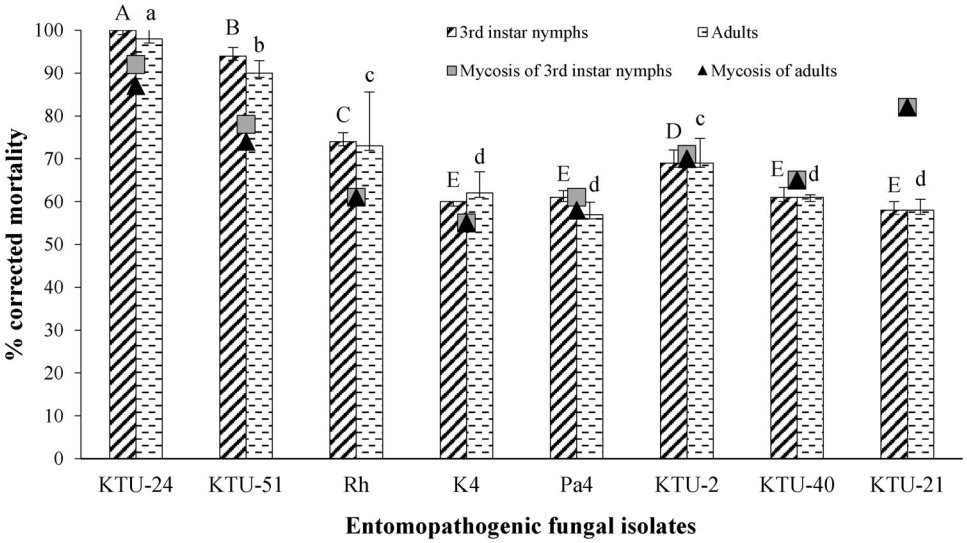

FIGURE 1 Screening test of entomopathogenic fungi on third instar nymphs and adults of the pest under laboratory condition at the concentration of 10^7 conidia ml^{-1}. Different case letters represent statistically significant differences amongst mortalities according to the least significant difference (LSD) multiple comparison test ($p < .05$). Mycosis indicates the sporulation rate in the moisture chamber after death. Mortality indicates the mean of three replications. Bars show the *SEM*

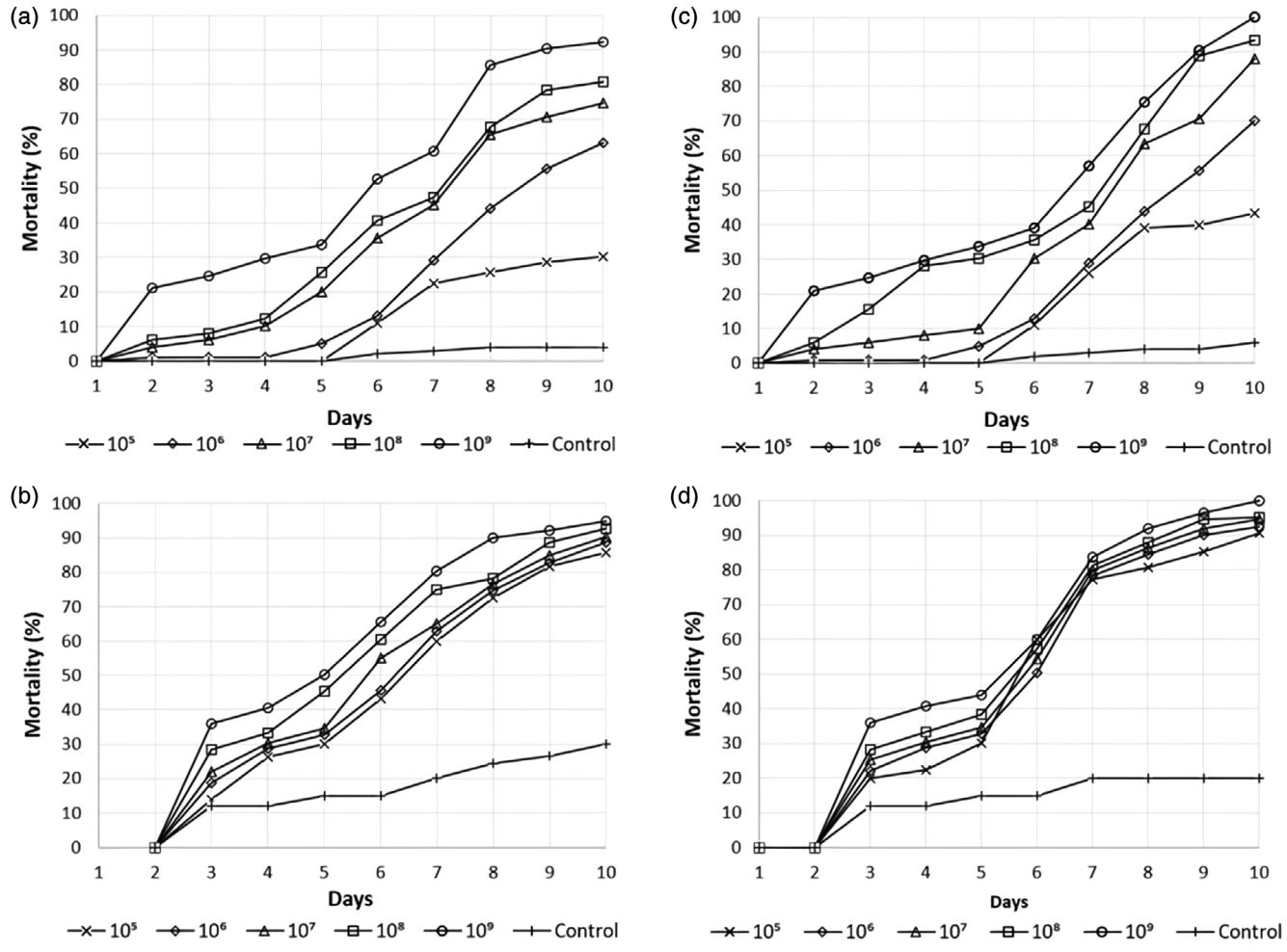

FIGURE 2 Dose–response experiments of KTU-24 on (a) third instar nymphs and (b) adults, KTU-51 on (c) third instar nymphs and (d) adults. Control: 0.01% Tween80. Mortality indicates the mean of three repetitions

differences between mortalities ($p > .05$). Mycosis on the cadavers exhibited that almost all insects were dead by fungal infection (Figure 3).

The effect of temperature on radial growth in vitro was determined by measuring the diameters of the colonies that were incubated at different temperatures. Different temperatures used in this

TABLE 2 Median lethal concentration (LC_{50}) of isolates on nymphs and adults of the pest

Stage	Isolate	LC_{50} (conidia ml^{-1}) (*FL*, %95)	Slope ± *SE*	LC_{95} (conidia ml^{-1})	*df*	X^2
Nymphs	KTU-24	6.08×10^5 ($2.5–12 \times 10^5$)	0.62 ± 0.048	2.3×10^9	3	5.076
	KTU-51	1.7×10^5 ($75–3.1 \times 10^5$)	0.66 ± 0.065	7.4×10^7	3	3.242
Adult	KTU-24	ND (ND-28)	0.15 ± 0.055	2.3×10^9	3	0.015
	KTU-51	ND (ND-1.9×10^2)	0.16 ± 0.072	2.3×10^6	3	3.516

Note: KTU-24: *B.bassiana*, KTU-51: *Metarhizium anisopliae*.
Abbreviations: *df*, degree of freedom; *FL*, fiducial limit; ND, not determined; *SE*, standard error; X^2, chi square.

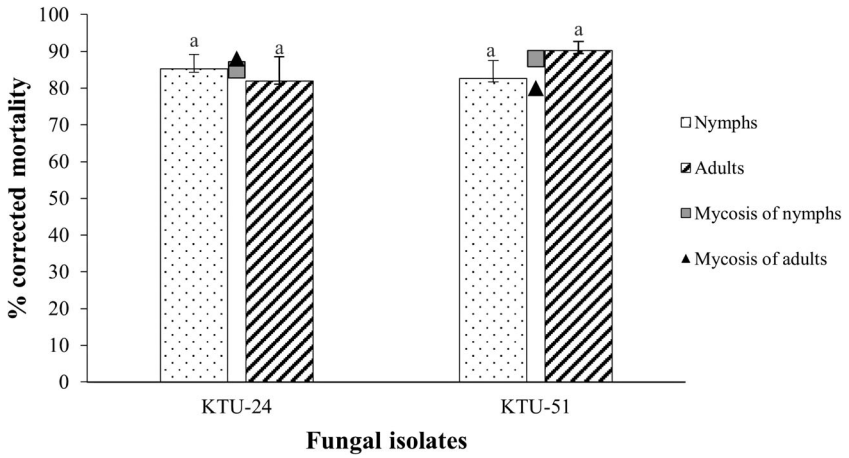

FIGURE 3 The efficacy of KTU-24 (*Beauveria bassiana*) and KTU-51 (*Metarhizium anisopliae*) on third instar nymphs and adults of the pest 20 days after application of 10^7 conidia ml^{-1} under semi-field condition. Mycosis indicates the percentage of sporulation in the moisture chamber after death. Different lower case letters represent statistically significant differences amongst mortalities according to the least significant difference (LSD) multiple comparison test ($p < .05$). Mortality indicates the mean of five replications. Bars show *SEM*

study had an effect on radial growth of both fungi ($F_{(3,16)} = 223.47$, $p = .000$) (Table 3). KTU-24 had the greatest diameter (24.76 ± 2.80 mm) at 28°C which is the control temperature, it enlarged slower at the other temperatures. On the other hand, KTU-51 showed best growth (20.0 ± 1.0 mm) at 20°C, its colony diameters decreased by increasing temperature. Neither of the isolates was grown at 37°C.

The effect of temperature on production of conidia was also determined by counting conidia after incubation at different temperatures. Different temperatures used in this study affected production of conidia in fungi ($F_{(3,16)} = 92.49$, $p = .000$). KTU-24 produced the highest number of conidia (23.3×10^8 conidia ml^{-1}) at 28°C as in the growth experiment. A severe decline occurred in production at 30°C. KTU-51 also produced the highest number of conidia (15.20×10^8 conidia ml^{-1}) at 28°C as did KTU-24 (Table 3). Since none of the isolates was grown at 37°C, production of conidia was not observed.

The effect of UV radiation on radial growth and production of conidia was determined by measuring the diameters of the colonies formed in media and by counting conidia after exposure to different temperatures and UV. It was determined that there are significant differences in UV-B compared to the control group in both radial growth ($F_{(1,36)} = 10.183$, $p = .003$) and conidia production ($F_{(2,61)} = 25.093$, $p = .000$). Each UV application affected radial growth of colonies and production of conidia in fungi under all tested conditions. Prolonged exposure to UV reduced radial growth and spores production of both fungi. These two isolates were observed to be affected by sudden radial growth restriction and spore production at 30°C (Table 4). Despite all this, both fungi succeed in radial growth and spore production to continue their life cycle.

Oil-based formulations of *B. bassiana* KTU-24 and *M. brunneum* KTU-51 isolates developed from powder spores including 10^9 spore per ml as mycoinsecticides, and they were named as RICANISIDAL *Bbas*-TR61 and RICANISIDAL *Met*-TR61, respectively.

Under laboratory conditions, all formulations caused over 80% mortality on both nymphs and adults (Figure 4). The insecticidal activity of all isolates, except Nostalgist-BL, on the third instar nymphs was not significantly different and over the 90% ($p > .05$). It was determined that *Bbas*-TR61 and *Met*-TR61 were the most effective formulations and yielded 96% mortality with 1×10^8 spore ml^{-1} concentration on third instar nymphs at 20 days. On the other hand, *Metarhizium* formulations (*Met*-TR61 and *Met*-52) showed statistically same mortality rate on the adults ($p > .05$), but efficacy higher than the *Beauveria* formulation ($p < .05$) (Figure 4).

Under semi-field conditions, *Bbas*-TR61 and *Met*-TR61 were tested on the first and the third instar of nymphs and adults and displayed over 97% mortality with 10^8 spore ml^{-1} concentration. However, Met52 and Nostalgist yielded very low mortality to compare with our products (Figure 5). In addition, it was displayed that insecticidal effects of our formulations were increased by increasing dose on both nymphs and adults and caused 100% mortality at the concentration of 10^9 spore $ml^{-§}$ on all stages of *O. japonica* (Figure 6).

TABLE 3 Effects of different temperatures on radial growth and number of conidia

	Radial growth (7 days PI, mm ± *SD*)		Number of conidia (14 days PI, spore/ml ± *SD*)	
Temperature (°C)	KTU-24	KTU-51	KTU-24	KTU-51
20	15.40 ± 0.20*b*	20.00 ± 1.00*a*	13.4 × 10^8 ± 2.20*b*	14.56 × 10^8 ± 1.33*a*
28	24.76 ± 2.80*a*	18.55 ± 1.89*a*	23.3 × 10^8 ± 2.68*a*	15.20 × 10^8 ± 3.99*a*
30	13.70 ± 0.20*b*	10.44 ± 2.56*b*	2.84 × 10^8 ± 0.31*c*	11.40 × 10^8 ± 2.52*a*
37	NG	NG	NG	NG

Note: Radial growth and number of conidia indicate the mean of three repetitions. Different case letters in a column represent statistically significant differences amongst the means according to the LSD multiple comparison test ($p < .05$).
Abbreviations: LSD, least significant difference; NG, no growth; PI, postinoculation; *SD*, standard deviation.

TABLE 4 Effect of UV-B on maximum growth diameters (mm ± *SD*) and conidia production (conidia/ml) ± *SD*) at various experimental temperatures

Isolate	20°C	Control (20°C)	28°C	Control (28°C)	30°C	Control (30°C)
	30' UV-B (mm ± *SD*)					
KTU-24	15.98 ± 0.02*b*	16.43 ± 0.01*a*	14.21 ± 0.31*b*	16.91 ± 0.96*a*	15.06 ± 0.08*a*	15.49 ± 0.05*a*
KTU-51	14.33 ± 0.46*b*	15.37 ± 0.00*a*	15.76 ± 2.51b	16.16 ± 3.31*a*	8.83 ± 0.24*b*	11.78 ± 0.12*a*
	60' UV-B (mm ± *SD*)					
KTU-24	14.83 ± 0,24*b*	15.92 ± 0.14*a*	13.45 ± 0.89*b*	16.91 ± 0.96*a*	14.83 ± 0.24*b*	15.32 ± 0.12*a*
KTU-51	13.83 ± 0.24*b*	15.11 ± 0.08*a*	14.34 ± 0.21*b*	16.16 ± 3.31*a*	8.39 ± 0.38*b*	10.21 ± 0.2*a*
	30' UV-B (conidia/ml × 10^8 ± *SD*)					
KTU-24	26.33 ± 0.14*a*	31.44 ± 0.10*b*	36.33 ± 0.59*b*	42.83 ± 0.76*a*	20.07 ± 0.10*b*	28.07 ± 0.11*a*
KTU-51	14.55 ± 0.07*a*	15.53 ± 0.05*b*	11.45 ± 1.26*b*	16.13 ± 2.31*a*	10.27 ± 0.03*b*	14.27 ± 0.02*a*
	60' UV-B (conidia/ml × 10^8 ± *SD*)					
KTU-24	34.90 ± 0.14*b*	38.70 ± 0.11*a*	36.21 ± 0.32*b*	42.83 ± 0.76*a*	2.57 ± 0.04*b*	15.57 ± 0.03*a*
KTU-51	15.75 ± 0.35*b*	16.08 ± 0.71*a*	12.13 ± 1.39*b*	16.13 ± 2.31*a*	1.56 ± 0.02*b*	9.17 ± 0.01*a*

Note: Radial growth and number of conidia indicate the mean of three replications. Control: no UV-B treatment. Different lower cases indicate the statistical differences between UV-B treated and non-treated fungus at a temperature.
Abbreviations: *SD*, standard deviation; UV, ultraviolet.

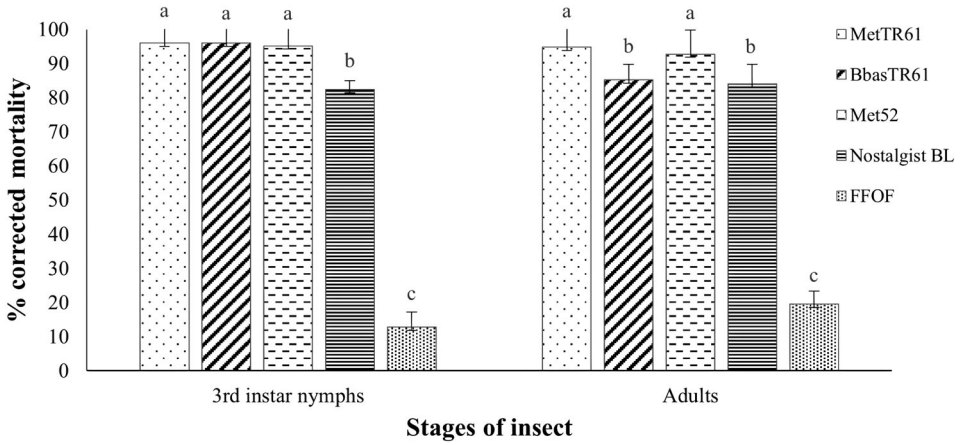

FIGURE 4 The efficacy of formulations against third instar nymphs and adult of the pest under laboratory conditions at the concentration of 10^8 conidia ml^{-1}. MetTR61: oil-based formulation of *Metarhizium anisopliae* KTU-51, BbasTR61: oil-based formulation of *Beauveria bassiana* KTU-24, Met52: *M. anisopliae* commercial formulation, Nostalgist-BL: *B. bassiana* commercial formulation. FFOF: Fungus free oil formulation. Different lowercase letters represent statistically significant differences amongst mortalities according to the least significant difference (LSD) multiple comparison test ($p < .05$). Bars show *SE*. Mortality indicates the mean of three replications

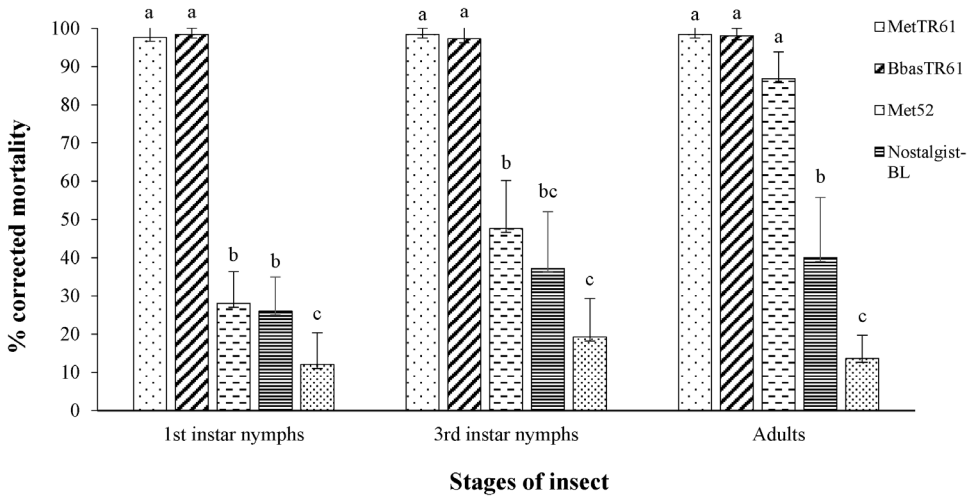

FIGURE 5 The efficacy of our oil-based formulations and commercial formulations under semi-field conditions at 10^8 conidia/ml against the different stages of insect. MetTR61: oil-based formulation of *Metarhizium anisopliae* KTU-51, BbasTR61: oil-based formulation of *Beauveria bassiana* KTU-24, Met52: *M. anisopliae* commercial formulation, Nostalgist-BL: *B. bassiana* commercial formulation. Different lowercase letters represent statistically significant differences amongst mortalities according to the least significant difference (LSD) multiple comparison test ($p < .05$). Bars show *SE*. Mortality indicates the mean of five replications

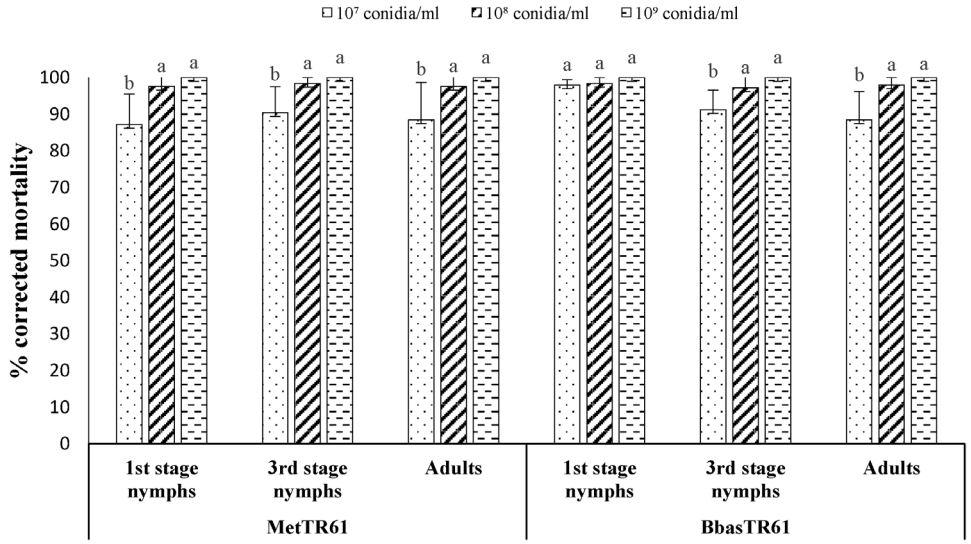

FIGURE 6 The efficacy of our formulations under semi-field conditions against the different stages of insect using different concentrations. MetTR61: oil-based formulation of *Metarhizium anisopliae* KTU-51, BbasTR61: oil-based formulation of *B. bassiana* KTU-24. Different lower-case letters represent significant differences amongst mortalities according to the least significant difference (LSD) multiple comparison test ($p < .05$). Bars show *SE*. Mortality indicates the mean of five replications

4 | DISCUSSION

The main goal of this study was to develop and implement innovative tools for sustainable management of *O. japonica* in Turkey. The priority is to offer new pest management strategies safe for human health and environment, in order to stimulate healthy tea production. The results of this study will enable farmers to produce healthier, locally grown tea. This in turn will contribute to an improved income for the farmer and help boost the local economy. For achieving this goal, entomopathogenic fungal isolates were tested under laboratory conditions against target insect. Two isolates with the highest insecticidal effect were tested under field conditions and developed as oil formulation. Finally, developed mycoinsecticides were applied on the pest under laboratory and semi-field conditions for the first time as an alternative to chemical control or as part of Integrated Pest Management programmes of *O. japonica*.

Until now, several studies have been carried out about isolation, characterisation and virulence of entomopathogenic fungi including *B. bassiana* and *M. brunneum* from insect and soil samples have been carried out in Black Sea Region in Turkey, which have great conditions for pest development (Kocacevik et al., 2015; Sevim, Demir, & Demirbag, 2010; Sonmez, Sevim, Demirbag, & Demir, 2016; Tanyeli, Sevim, Demirbag, Eroglu, & Demir, 2010). It was determined that the host ranges of entomopathogenic fungal isolates obtained from these studies include different species of Coleoptera, Lepidoptera, Hemiptera, Orthoptera, Hymenoptera, and they had significant insecticidal effects on various pests that live in the same region (Sevim, Demir, Tanyeli, & Demirbag, 2010; Sevim, Demir, Sonmez,

Kocacevik, & Demirbag, 2013; Kocacevik, Sevim, Eroglu, Demirbag, & Demir, 2016).

In the first stage, the selection of the most suitable isolate is critical for the development of mycoinsecticide. In fact, genetic variability is amongst the main factors influencing fungus pathogenesis in the host (Berón & Diaz, 2005; Chouvenc, Su, & Robert, 2009; Talwar, 2005; Xiao et al., 2012). Therefore, effective isolate selection in the laboratory improves the chances of success in the field. In addition, the high effectiveness of microbial factors to be used in biological control is related to the adaptation of the factors to the conditions of the region. Application of entomopathogenic fungi in the region where they were isolated had been found to give better results than entomopathogenic fungus species obtained from other countries and regions (Berón & Diaz, 2005). Fungal isolates used in this study are indigenous isolates isolated from the tea region and identified in previous studies (Sevim, Demir, & Demirbag, 2010; Sevim, Demir, Tanyeli, & Demirbag, 2010).

In the present study, eight entomopathogenic fungal isolates including *Beauveria* and *Metarhizium* genera were used to determine most effective fungal isolate against *O. japonica* nymphs and adults under laboratory conditions. Screening and dose response experiments showed that *B. bassiana* KTU-24 and *M. brunneum* KTU-51 isolates were the most promising isolates. Also, these isolates showed high mortality (82–90%) during the field experiments. These species are the most widely used biological control agents that can suppress a variety of economically important agricultural and forestry pests compared to other entomopathogenic fungi (Lacey & Shapiro-Ilan, 2008). Entomopathogenic fungi from local isolates of *Beauveria* and *Metarhizium* genus were tested on the pest for the first time with our study except for Gokturk et al. (2018) recorded that *B. bassiana* ET 10 isolate had 20 and 6% mortalities against *O. japonica* nymphs and adults, respectively.

To our knowledge, only three studies used entomopathogenic fungi as biocontrol agent against *O. japonica* in Turkey (Ak et al., 2014; Gokturk et al., 2018; Güçlü et al., 2010). In these studies, 10^6 and 10^7 conidia ml^{-1} concentrations were used in both laboratory and field trials. The applications were carried out on different plants, tea and tomato in the laboratory and kiwi and apple in the field conditions. Their results demonstrated that *L. muscarium* and *C. coronatus* had a potential control against this pest. Although biological activities were determined in that study, any product was not developed from that. Therefore, the prototype mycoinsecticides from *B. bassiana* and *M. brunneum* species, which can be used for the control of *O. japonica*, were developed for the first time in the present study and tested under laboratory and field conditions.

Microclimatic factors such as temperature and relative humidity are important in improving the efficacy of fungal treatment in the field. In Rize province of Turkey, total rainfall (July and August 2018) of 173.35 mm with daily average of 5.6 mm and 80% humidity, and temperatures between 15 and 35°C were conducive for fungal growth. In this study, the optimum temperature of KTU-24 and KTU-51 isolates was determined as 28 and 20°C, respectively. The KTU-51 isolate showed the best radial growth at 20°C. However, increasing temperature caused reduction in radial growth of KTU-51 (Table 3). Dimbi, Maniana, Luz, and Mueke (2004) determined that the optimum temperature range for *M. anisopliae* isolates was between 24 and 30°C. In general, the optimum temperature for the growth and germination of entomopathogenic fungi in vitro condition is 25°C. However, the germination and growth rate are decreased or deteriorating rapidly over 30°C (Fransen, Winkelman, & Van Lenteren, 1987). Qazzaz, Al-Masri, and Barakat (2015) and Ortucu and Algur (2017) reported that radial growth and spore production of five *B. bassiana* isolates at different temperatures started to decrease especially at 30°C. The radial growth of *B. bassiana* KTU-24 decreased significantly when the temperature exceeded 28°C (Table 3). Also, Kessler, Matzke, and Keller (2003) and Cheong (2015) showed that a temperature above 27–30°C inhibited mycelial growth and killed spores. The fact that strains belonging to *B. bassina* species can develop in a wide temperature range such as 8–35°C confirms the study of Fargues et al. (1997). Fernandes, Rangel, Moraes, Bittencourt, and Roberts (2008) determined that there is a connection between the geographic origin and cold tolerance of *B. bassiana* isolates, but this connection reported that it is not seen in heat tolerance. The ability of entomopathogenic fungi to grow and sporulate under wide temperature ranges is very useful in their application as biological control agents, particularly in semi-arid climates. In addition, solar UV radiation is perhaps the most harmful environmental factor affecting the viability of fungal spores (Moore, Bridge, Higgins, Bateman, & Prior, 1993). Ortucu and Algur (2017) also reported that there are sudden changes in both radial and spores production, especially in 30 and 60 min of UV-B application, as in our study. Oil-based formulations are reportedly able to significantly increase the tolerance of conidia to UV radiation, with improved germination of conidia, in comparison in traditional water-based formulations (Alves, Bateman, Prior, & Leather, 1998). In the present study, both fungal isolates affected by UV, they were able to grow and sporulate. A suitable formulation benefits the application and handling of the bio-agent, and increases its efficacy by protecting the active ingredient from adverse environmental factors (Nian et al., 2015).

Mycopesticides have been conventionally formulated and marketed as products to be applied on insect pests in many crops (Faria & Wraight, 2007). Some formulations provide protection against abiotic factors that are deleterious to spores. For example, oil-based formulations can protect spores against imbibitional damage (Xavier-Santos, Lopes, & Faria, 2011) and the detrimental effect of UV (Hedimbi et al., 2008) or chemical pesticides (Lopes, Pauli, Mascarin, & Faria, 2011). All these features indicate that oil-based formulations are becoming important today. The advantages of oil-based formulations, including killing effect and protection against negative environmental effects, have been widely informed in the control against pests (Lopes et al., 2011; Oliveira, Lopes, Rezende, & Delalibera Jr., 2018). Nevertheless, few entomopathogenic fungi are commercially present in the market or have been applied in the field (Oliveira et al., 2018).

The entomopathogenic fungi formulated in the oils show increased efficiency than the spores formulated in the water or any

other carrier (Lomer et al., 1993; Lomer, Prior, & Kooyman, 1997). The situation warrants shift to use of oil-formulation of fungi which showed good results in biological control of insect pests under field conditions (Feng, Poprawski, & Khachatourians, 1994). When we examine the literature, oil formulations were tested in different insect groups and in different proportions. For example, formulations of *M. anisopliae* and *B. bassiana* have been used against *Sitophilus oryzae*, *Rhyzopertha dominica* (Batta, 2008), *Anopheles gambiae* and *Anopheles stephensi* (Bukhari, Takken, & Koenraadt, 2011) as biocontrol agents. Also, different oil formulations such as 50% oil formulations of *B. bassiana* (Luz & Batagin, 2005); 10% coconut oil formulations and 20% oil formulations of *M. anisopliae* against ticks (Hedimbi, Kaaya, & Chinsembu, 2011); 19% coconut oil and 28% soybean oil formulations of *B. bassiana* against almond bark beetles (Batta, 2007) and 50% rapeseed and camellia oil formulations of *Zoophthora radicans* against *Plutella xylostella* (Batta, Rahman, Powis, Baker, & Schmidt, 2011) are known.

Our study is the first contribution to the development of an oil-based formulation of *B. bassiana* and *M. brunneum* as a biological control tool against *O. japonica*, the pest of many plants in Black Sea Region. We concluded by testing the fungal products developed in this study and commercial products against the nymphs and adults of the pest under laboratory and field conditions. In all trials in the field, tea was used as the host plant. As a result of this study, two prototype mycoinsecticides were produced for the first time in Turkey against *O. japonica*. Both products (RICANISIDAL *Bbas*-TR61 and RICANISIDAL *Met*-TR61) are also first biopesticides against *O. japonica* in the world. Products developed from entomopathogenic fungi had an incomparably higher effect on pests than commercial mycoinsecticides (*B. bassiana* and *M. anisopliae*) used as control. These two commercial products, known to the whole world, are of course not licensed against these pests, but they have been used for control of various other pests for long times (Abdel-Raheem, 2019; Jaronski & Mascarin, 2017). Our products have 90% and above mortality indicated that their virulence was higher than commercial mycoinsecticides.

In summary, it has been demonstrated through this study that oil-based fungal formulations of *B. bassiana* and *M. brunneum* kill both *O. japonica* nymphs and adults effectively within approximately 10 and 20 days in the laboratory and field conditions, respectively. The fact that there is no literature to compare with this study for the development of mycoinsecticide makes this study important in terms of being a reference study in control of this pest. Thus, the use of BbasTR61 and MetTR61 mycoinsecticides to control *O. japonica* is suggested to increase the chances of pest control. In the future, monitoring plan for the pest will be developed and recommendations will be provided to tea growers regarding sustainable management of *O. japonica*. The results of the study will be shared with private and public institutions for the licensing, commercialisation and use of products.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Seda Biryol https://orcid.org/0000-0003-0881-5004
Ebru Güney https://orcid.org/0000-0001-9347-0359
Ardahan Eski https://orcid.org/0000-0002-9621-2854
Zeynep Bayramoğlu https://orcid.org/0000-0001-6994-1106
Kazım Sezen https://orcid.org/0000-0002-2903-0460
Zihni Demirbag https://orcid.org/0000-0001-5487-1977
İsmail Demir https://orcid.org/0000-0001-6227-0039

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