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Pathogenicity of selected entomopathogenic fungal isolates against the oak lace bug, *Corythucha arcuata* Say. (Hemiptera: Tingidae), under controlled conditions

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Abstract: *Corythucha arcuata* (Say) (Hemiptera: Tingidae) is a pest of oaks that is highly distributed in several regions of the world, including North America, the Balkan Peninsula, Europe, and Turkey. In the present study, ten entomopathogenic fungi belonging to four different genera (*Metarhizium* (×4), *Beauveria* (×4), *Isaria* (×1), and *Myriodontium* (×1)) were tested against nymphs and adults of *C. arcuata* under laboratory conditions. Based on the initial screening studies, it was shown that all isolates were able to infect both nymph and adult individuals of the pest after application of 1×10^7 mL^{-1} conidial concentration. Among the tested isolates, *Beauveria bassiana* KTU-24 caused the highest mortality against both nymphs and adults within 14 days, with 80% and 90% mortality, respectively. This isolate also produced the highest mycosis values on both nymphs and adults, with 77% and 83% mycosis, respectively. Therefore, this isolate (*B. bassiana* KTU-24) was selected for concentration–mortality response tests for further characterization. The concentration–mortality response tests showed that the conidial concentrations of 1×10^8 and 1×10^9 mL^{-1} of *B. bassiana* KTU-24 produced 100% mortality against nymphs and the conidial concentration of 1×10^9 mL^{-1} of this isolate caused 100% mortality against adults. Based on probit analysis, the LC_{50} values of the isolate *B. bassiana* KTU-24 were calculated as 1.17×10^7 and 6.44×10^6 conidia mL^{-1} against nymphs and adults, respectively. This study indicates that *B. bassiana* KTU-24 has a significant potential for further investigation as biological control agent against *C. arcuata*.

Key words: *Corythucha arcuata*, entomopathogenic fungi, *Beauveria bassiana*, lethal concentration

1. Introduction

The oak lace bug, *Corythucha arcuata* (Say) (Hemiptera: Tingidae), is one of the most important pests of oak trees worldwide, especially on *Quercus acuminate* (M. Martens & Galeotti) (Fagales: Fagaceae), *Q. alba* (L.), *Q. macrocarpa* (Michx.), *Q. muehlenbergii* (Engelm.), *Q. prinoides* (Willd.), *Q. prinus* (L.) and *Q. rubra* (L.). This pest is also occasionally found on plants belonging to the genera *Castanea*, *Acer*, *Pyrus*, *Malus*, and *Rosa* (Drake and Ruhoff, 1965; Drew and Arnold, 1977). Until 2000, *C. arcuata* was known to exist only in North America. Later, this species was reported to exist in northern Italy (Bernardinelli and Zandigiacomo, 2000), Turkey (Mutun, 2003), and Switzerland (Forster et al., 2005). A recent study showed that this species has been also reported in Bulgaria and the Balkan Peninsula (Dobreva et al., 2013). Both nymphs and adults of this pest feed on leaves of oak trees, causing chloric discoloration (Dobreva et al., 2013). Infested leaves have a whitish blotching or stippling on the upper surface and dark brown spots of excrements and groups of bug eggs on the lower surfaces. The sucking up of the cellular sap material reduces photosynthesis and respiration in host plants and also affects the aesthetic value of the trees. In cases of heavy infestation, the pest can cause defoliation (Mutun et al., 2009).

Corythucha arcuata is also under consideration by the European and Mediterranean Plant Protection Organization (EPPO) and listed in the Alert List. There are a few control methods for *Corythucha arcuata.* These include planting shrubs or small trees in sunny dry sites and spraying oil or contact insecticides on the underside of leaves. Alternatively, chemical control is sometimes used in North America on ornamental oaks (Sparks et al., 1994). In urban environments, where people generally do not like the use of chemicals, it is possible to apply some natural enemies against *Corythucha* spp. There are several natural enemies of the oak lace bug such as assassin bugs, minute pirate bugs, lacewings, spiders, and predaceous mites. However, they have not been used in any biocontrol programs to date. Before the oak lace bug exceeds the economic injury threshold, the development of control strategies is urgently required. These control strategies

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should be effective and environmentally friendly and allow the enhancement of natural enemies of the oak lace bug.

To date, many pathogens have been isolated from insects and their insecticidal effects have been determined on various agricultural and forest pests (Sevim et al., 2010a; Tanyeli et al., 2010; Danismazoglu et al., 2012; Demir et al., 2013; Erbaş et al., 2014; Imoulan and Elmeziane, 2014; Kocacevik et al., 2015). Some entomopathogenic fungi are among the most virulent pathogens used for controlling flying insects (Goettel et al., 1990; Sevim et al., 2010b, 2010c; Garrido-Jurado et al., 2011). In a similar study, Sevim et al. (2013) tested 13 entomopathogenic fungal isolates against another lace bug (*Corythucha ciliata*) and they found that *Beauveria bassiana* KTU-24 was a promising candidate as a fungal biocontrol agent against it.

Despite the rapid dispersion of *C. arcuata* throughout Turkey (Mutun et al., 2009), no study has been reported about the application of entomopathogenic fungi against this pest. In the present study, we tested 10 entomopathogenic fungi against both nymphs and adults of *C. arcuata* under laboratory conditions to find the most effective one as a possible entomopathogenic fungal biocontrol agent. This is the first study to demonstrate the efficacy of entomopathogenic fungi against *C. arcuata*. The results presented here can be beneficial in future biological control programs for *C. arcuata*.

2. Materials and methods

2.1. Collection of insects

Nymphs and adults of *Corythucha arcuata* were collected from infested *Quercus robur* (L.) (Fagales: Fagaceae) trees in the vicinity of Trabzon, Turkey, between June and August 2013. They were carefully collected from the undersides of leaves with a soft fine-tipped paintbrush or sometimes by taking whole leaves. For flying adults, a sweep net was used. Insect and leaf samples were placed into plastic boxes (20 × 20 cm) with ventilated lids and transported to the laboratory. Healthy nymphs and adults were acclimated for 2 days to the laboratory conditions and then the healthy insects were selected for bioassays.

2.2. Fungal cultures

The fungal isolates used in this study were obtained from stock cultures at the Microbiology Laboratory of the Department of Biology at Karadeniz Technical University, Trabzon, Turkey. The entomopathogenic fungi consisted of *Metarhizium anisopliae* sensu lato (4 isolates), *Beauveria bassiana* (Bals.) Vuill. (3 isolates), *B. pseudobassiana* S.A. Rehner & R.A. Humber (1 isolate), *Isaria fumosorosea* Wize (1 isolate), and *Myriodontium keratinophilum* Samson & Polon (1 isolate) (Table 1) and were initially plated on PDAY (potato dextrose agar + 1% yeast extract; Merck, Darmstadt, Germany) for 1 week at 28 ± 1 °C under 12:12 photoperiod. After that, all isolates were streaked with a loop on PDAY and incubated at 28 ± 1 °C for 4–5 days under 12:12 photoperiod to obtain fungal colonies propagated from single conidia. After incubation, a single colony for each isolate was transferred to another fresh PDAY plate and incubated at 25 ± 1 °C for 4 to 5 weeks until the plates were fully overgrown. The fully sporulated cultures were used for bioassays.

2.3. Conidial suspensions and spore viability

Conidial suspensions of the fungal isolates were prepared from previously incubated 4-week-old petri dishes for each isolate. Ten milliliters of 0.01% sterile Tween 80

Table 1. Fungal isolates used in this study and their sources.

No.	Species	Isolates	Source	Reference
1	*Metarhizium anisopliae*	KTU-60	Soil	Sevim et al. (2010b)
2	*M. anisopliae*	KTU-2	Soil	Sevim et al. (2010b)
3	*M. anisopliae*	Gg-7	*Gryllotalpa gryllotalpa* (L.) (Orthoptera: Gryllotalpidae)	Sonmez et al., 2016
4	*M. anisopliae*	Gg-12	*G. gryllotalpa*	Sonmez et al., 2016
5	*Beauveria bassiana*	KTU-24	*Thaumetopoea pityocampa* (Den. & Schiff.) (Lepidoptera: Thaumetopoeidae)	Sevim et al. (2010b)
6	*B. bassiana*	KTU-7	Soil	Sevim et al. (2010b)
7	*B. bassiana*	Gg-1	*G. gryllotalpa*	Sonmez et al., 2016
8	*B. pseudobassiana*	KTU-53	Soil	Sevim et al. (2010b)
9	*Isaria fumosorosea*	KTU-42	Soil	Sevim et al. (2010b)
10	*Myriodontium keratinophilum*	Gg-11	*G. gryllotalpa*	Sonmez et al., 2016

(Applichem, Darmstadt, Germany) was added to each petri dish and the conidia from the surface of the agar plates was gently scraped by a sterile stainless steel triangle-headed cell spreader (20 cm). After that, the obtained conidial suspensions were filtered through 2 layers of sterile cheesecloth into 50-mL sterile plastic tubes (Falkon) to remove mycelium and agar pieces. Conidial suspensions were vortexed for 5 min for homogenization. Finally, the spore concentration was determined with a Neubauer hemocytometer and the suspension was adjusted to the desired concentration.

Conidial viability was assessed by determining the percentage of the germinated conidia in 24 h after spreading 50 µL of conidial suspensions (1×10^6 mL^{-1}) on PDAY medium. Plates were incubated at 25 ± 1 °C under a 12:12 photoperiod. Conidia with germ tubes longer than their width or diameter were considered to have germinated. To estimate the percentage germination, a total of 300 conidia were examined from each treatment (100 per replicate), and 25 germinated conidia from each replicate plate were randomly chosen for measurement of germ tube length. Isolates that had 95% or higher germination rates were used for bioassays.

2.4. Screening tests

A total of 10 fungal isolates were tested against both nymphs and adults of *C. arcuata* to evaluate their virulence. Conidial suspensions of fungal isolates were prepared as described above. For the screening test, 10 of the same stage nymphs and adults for each isolate and replicate were carefully brushed from the undersides of leaves with a soft paintbrush into plastic petri dishes (90 × 16 mm) and the conidial suspensions (1×10^7 conidia mL^{-1}) were applied to insects with a sterile sprayer (100 mL) for 10 s. After that, alive sprayed nymphs or adults were separately placed on the undersides of small oak leaves in a plastic box (20 × 20 cm) with a ventilated lid. Ten nymphs and adults for each replicate were used as controls and were only sprayed with sterile distilled water with Tween 80 (0.01%). All experiments were repeated three times at different times. All plastic boxes containing sprayed nymphs and adults were kept in rearing boxes at 25 ± 1 °C and 70 ± 5% relative humidity for 2 weeks under a 12:12 h (L:D) photoperiod. Treated nymphs and adults were fed with freshly collected and washed oak leaves for 2 weeks and were checked daily. During the incubation period, dead nymphs and adults were counted and cadavers were immediately surface sterilized with 1% sodium hypochlorite for 30 s, followed by 3 rinses with sterile distilled water. After that, they were put into the moisture chamber to stimulate fungal sporulation outside the cadaver. Finally, the effectiveness of the isolates was measured by determination of mortality rates and calculation of percentage of mycoses by using Abbott's formula (Abbott, 1925).

2.5. Concentration–mortality response tests

Beauveria bassiana KTU-24 was selected for the concentration–mortality response test based on its high efficacy against both nymphs and adults of *C. arcuata* in the screening test. Conidial suspensions of fungal isolates were prepared as described above and a series of dilutions was prepared: 1×10^6, 1×10^7, 1×10^8, and 1×10^9 conidia mL^{-1}. Ten healthy nymphs and adults for each replicate were treated with 4 different conidial concentrations by using a sterile sprayer (100 mL). After that, alive sprayed nymphs or adults were separately placed on the underside of small oak leaves in a plastic box (20 × 20 cm) with a ventilated lid. Ten nymphs and adults for each replicate were used as controls and were sprayed with sterile distilled water with Tween 80 (0.01%). The boxes were closed to keep moisture in and were maintained at 25 ± 1 °C and 70 ± 5% relative humidity for 2 weeks under a 12:12 h (L:D) photoperiod. All experiments were repeated three times at different times. Mortality of nymphs and adults was checked daily over 15 days of bioassays. Finally, the mortality data were corrected using Abbott's formula (Abbott, 1925) and the lethal concentration (LC_{50}) value was calculated using probit analysis.

2.6. Statistical analysis

The mortality data of isolates on nymphs and adults of *Corythucha arcuata* were corrected using Abbott's formula (Abbott, 1925) and percentages of mycosed insect cadavers were calculated. For the screening tests, the data were subjected to analysis of variance (ANOVA) and later to Dunnett's one-tailed t-test to compare test isolates against the control group with respect to mortality and mycosis ($P < 0.05$). Moreover, the data were subjected to ANOVA and later to least significant difference (LSD) multiple comparison tests to compare isolates with each other in terms of mortality and mycosis ($P < 0.05$). For the concentration–mortality response tests, the data were subjected to ANOVA and later to LSD multiple comparison test to compare different concentrations ($P < 0.05$). Before performing the ANOVA, all data set were tested for homogeneity of variance using Levene's statistic. Difference between nymph and adult mortality was evaluated by chi-squared test ($P < 0.05$). Finally, LC_{50} values were determined by probit analysis. Computations for all experiments were performed using SPSS 16.0.

3. Results

3.1. Screening bioassay

Fungal isolates caused different mortalities in both nymphs and adults in the screening tests (Figures 1 and 2). In the case of nymphs, the mortalities changed depending on the isolate and there was a significant difference among isolates in terms of mortality ($F = 4.02$, $df = 9$, $P < 0.05$). The highest mortality was recorded from *B. bassiana* KTU-24 with 80% within 14 days after inoculation. The second

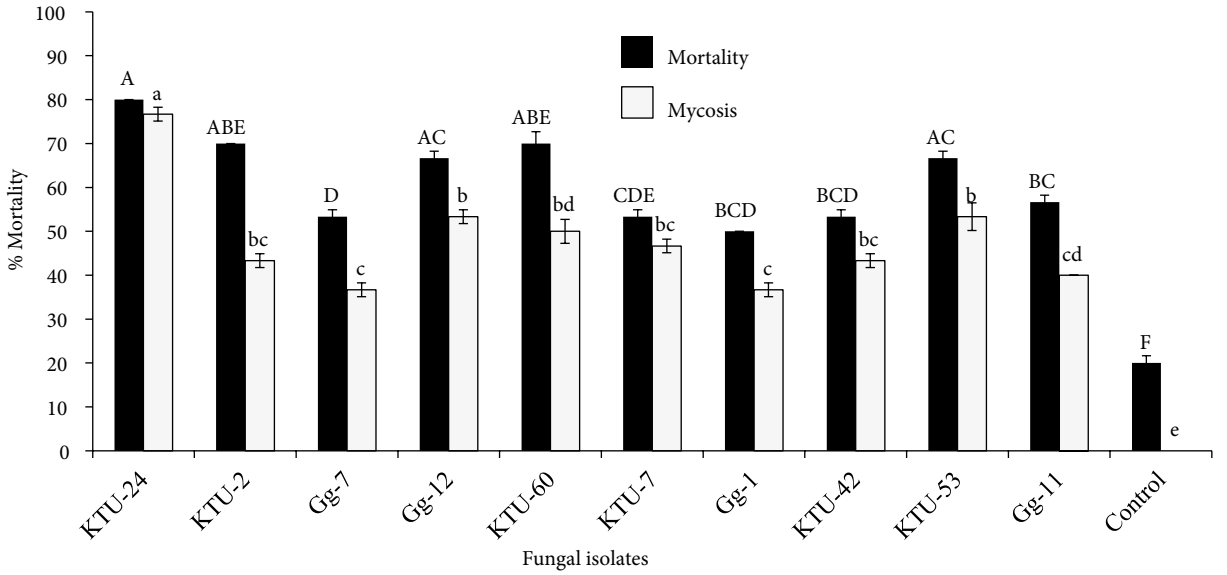

Figure 1. Virulence of fungal isolates against *Corythucha arcuata* nymphs within 14 days of application of the conidial concentration of 1×10^7 mL^{-1}. Mortality data were corrected according to Abbott's formula (Abbott, 1925). Different uppercase and lowercase letters represent statistically significant differences among treatments according to LSD multiple comparison test in terms of mortality and mycosis, respectively ($P < 0.05$). Bars show standard deviation.

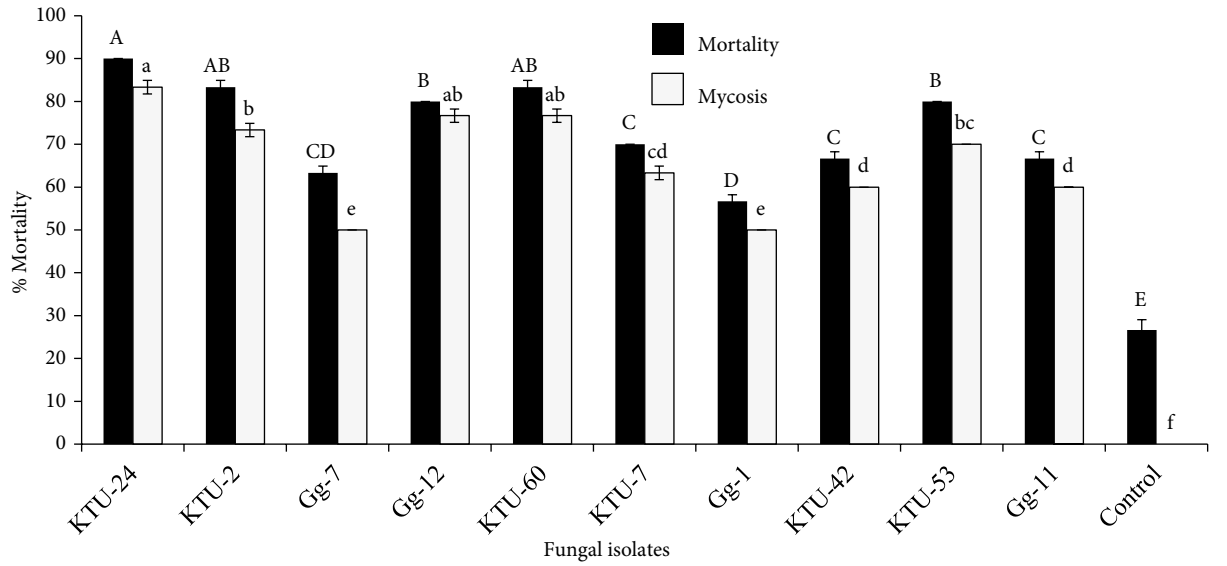

Figure 2. Virulence of fungal isolates against *Corythucha arcuata* adults within 14 days of application of the conidial concentration of 1×10^7 mL^{-1}. Mortality data were corrected according to Abbott's formula (Abbott, 1925). Different uppercase and lowercase letters represent statistically significant differences among treatments according to LSD multiple comparison test in terms of mortality and mycosis, respectively ($P < 0.05$). Bars show standard deviation.

highest mortality was obtained from *M. anisopliae* KTU-2 and *M. anisopliae* KTU-60 with 70% within the same period (Figure 1). All isolates produced different mycoses in comparison to each other ($F = 8.92$, $df = 9$, $P < 0.05$) and there was a significant difference between the control and fungal isolates with respect to mycosis ($F = 23.64$, $df = 10$, $P < 0.05$). *B. bassiana* KTU-24 produced the highest mycosis value with 76%, which was different from all other treatments and the control ($P < 0.05$). The second highest mycosis was obtained from *M. anisopliae* Gg-12 and *B. pseudobassiana* KTU-53 with 53% ($P < 0.05$) (Figure 1).

In the case of adults, all isolates also produced different mortality values in comparison to each other ($F = 17.33$, $df = 9$, $P < 0.05$) and there was a significant difference between the control and treatments ($F = 40.6$, $df = 10$, $P < 0.05$). The highest adult mortality was also obtained

from *B. bassiana* KTU-24 with 90% within 14 days of application. The second highest mortality was recorded from *M. anisopliae* KTU-2 and *M. anisopliae* KTU-60 with 83% within the same period (Figure 2). All isolates also produced different mycosis values in comparison to each other ($F = 23.75$, $df = 9$, $P < 0.05$) and there was a significant difference between the control and treatments with respect to mycosis ($F = 102.72$, $df = 10$, $P < 0.05$). The highest mycosis value was also obtained from *B. bassiana* KTU-24 with 83% and the mycosis values of other isolates ranged from 50% to 77% (Figure 2).

We also found that there was a significant difference between nymphs and adults with respect to susceptibility to fungal isolates ($P < 0.05$). Nymphs were found to be more susceptible than adults.

3.2. Concentration–mortality response test

The concentration–mortality relationship was studied with *Beauveria bassiana* KTU-24 based on its high virulence on *C. arcuata* nymphs and adults. Four different concentrations (1×10^6, 1×10^7, 1×10^8, and 1×10^9 conidia mL^{-1}) were used in the concentration–mortality response test. In the case of the nymph test, mortality reached 100% within 7 days of application of 1×10^9 conidia mL^{-1} (Figure 3). There was a significant difference among concentrations and different concentrations of conidia affected the mortality of nymphs differently ($F = 62.33$; $df = 3, 12$; $P < 0.05$). In the case of the adult test, mortality reached 100% within 9 days of application of 1×10^9 conidia mL^{-1} (Figure 4). There was a significant difference among concentrations and different concentrations of conidia affected the mortality of nymphs differently ($F = 82.44$; $df = 3$; $P < 0.05$).

The LC_{50} values of isolate *B. bassiana* KTU-24 were calculated as 1.17×10^7 and 1.2×10^6 conidia mL^{-1} against nymphs and adults based on probit analysis, respectively (Table 2).

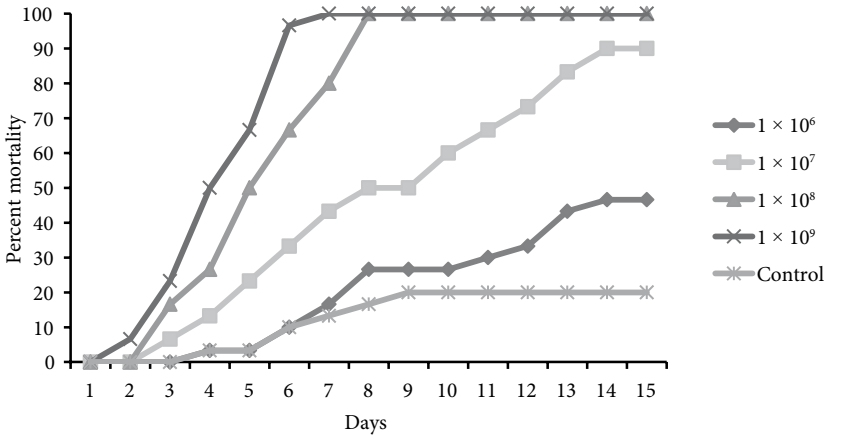

Figure 3. Cumulative mortality of *Corythucha arcuata* nymphs after application of various concentrations of *B. bassiana* KTU-24. Concentration unit is conidia mL^{-1}.

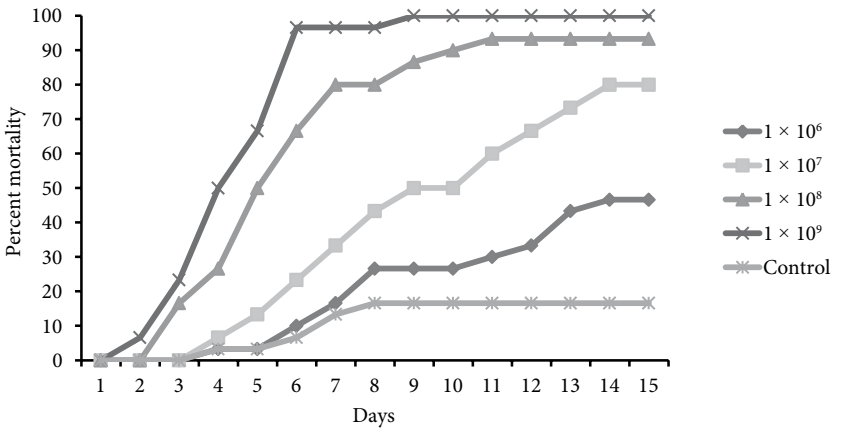

Figure 4. Cumulative mortality of *Corythucha arcuata* adults after application of different concentrations of *B. bassiana* KTU-24. Concentration unit is conidia mL^{-1}.

Table 2. Probit analysis parameters from the multiple-concentration bioassays performed with *B. bassiana* KTU-24 against nymphs and adults of *Corythucha arcuata*.

Stages	Intercept	LC_{50} (95% fiducial limits)	Slope ± SE (standard error)[a]	*df*	X^{2b}
Nymphs	–7.28 ± 1.15	1.17×10^7 (5.9×10^6–2.22×10^7)	1.03 ± 0.16	2	1.34
Adult	–6.5 ± 1.13	6.44×10^6 (2.8×10^6–1.26 10^7)	0.95 ± 0.16	2	1.59

a Slope of the concentration ± standard error response of adult and nymph of *C. arcuata* to *B. bassiana* KTU-24.
b Pearson chi-square goodness-of-fit test on the probit model (α = 0.05).

4. Discussion

Because of the undesired side effects of chemical insecticides on the environment and nontarget species, scientists have been looking at the possibility of developing a nonchemical pest control method for controlling pest populations in both agriculture and forestry. An alternative method to substitute chemical pesticides is the utilization of microbial control agents that are natural enemies of the insect pests with no harmful effects on mammals or the environment (Khetan, 2001). The use of microbial agents such as viruses, bacteria, fungi, nematodes, and protozoa and their products against insect pests is one of the most accepted approaches worldwide (Charles et al., 2000; Lacey et al., 2001; Uygun et al., 2010). Considering the environmental conditions of the oak lace bug's distribution areas in Turkey and the biological features of this pest, it is seen that the use of entomopathogenic fungi against *C. arcuata* could be the most appropriate approach (Sevim et al., 2012, 2013). Although some control methods against *C. arcuata* have been used around the world, this pest has been continuing to spread rapidly and cause damage to oak trees. In the present study, we determined that 10 entomopathogenic fungi isolated from different sources had different levels of virulence on *C. arcuata* ranging from 50% to 90% depending on different development stages of *C. arcuata*. Among the tested isolates, *Beauveria bassiana* KTU-24 was determined as the most virulent isolate for the nymphs and adults of *C. arcuata*.

Previously, some researchers showed that *Beauveria* spp. are natural fungal pathogens of various insect pests in agriculture, forestry, medicine, and veterinary medicine (Goettel et al., 1990, 2005; Kreutz et al., 2004; Sevim et al., 2010a, 2013). In particular, it has been shown that some species of this genus such as *B. bassiana*, *B. pseudubassiana*, and *B. caledonica* have a great potential for the control of various forest pests. Quesada-Moraga et al. (2006) reported that adults and pupae of the Mediterranean fruit fly were susceptible against *B. bassiana* isolates and mortality rates produced by these isolates ranged from 38% to 100% and 0% to 94.5%, respectively. Kocacevik et al. (2015) showed that *B. pseudubassiana* ARSEF9271 caused 100% mortality against both larvae and adults of *Dendroctonus micans* (Kug.) (Coleoptera: Curculionidae). Glare et al. (2008) reported that two important forest beetles (*Hylastes ater* (Paykull) and *Hylurgus ligniperda* (Fabricius) (Curculionidae: Scolytinae)) were attacked by *B. caledonica* under natural conditions. Sevim et al. (2010a) showed that entomopathogenic fungi belonging to several species produced different mortality values against *Thaumetopoea pityocampa* Schiff. (Lepidoptera: Thaumetopoeidae). Among these isolates, *B. bassiana* KTU-24 caused 100% mortality and mycosis values against larvae of this pest. Sevim et al. (2013) tested 13 different entomopathogenic fungi against another lace bug (*Corythucha ciliata* (Say) (Hemiptera: Tingidae)) under laboratory conditions. Among the tested isolates, *B. bassiana* KTU-24 was found to be the most effective. Finally, Sevim et al. (2010c) also showed that *B. bassiana* KTU-24 caused 96% and 56% mortality values against larvae and adults of *D. micans* under laboratory conditions, respectively. All these studies suggest that some species of *Beauveria* spp. (especially *B. bassiana* and *B. pseudobassiana*) can be used against some forest pests in Turkey's forest for the control of important insect pests. In particular, *B. bassiana* KTU-24 seems to have great potential for the control of different forest pests such as *T. piyocampa*, *D. micans*, *C. ciliata*, and *C. arcuata* in Turkey.

We also determined that *C. arcuata* nymphs are more susceptible than adults in terms of susceptibility to fungal isolates ($P < 0.05$). All development stages in any insect's life cycle are not susceptible to fungal infection at the same rate (Inglis et al., 2001). Generally, early instar larvae or nymphs are more susceptible than older stages (Butt, 2002). This may be due to older instars having thicker cuticles than early instars. However, in some situations, older instar larvae are more susceptible than early instar larvae. For example, the 1st instar larvae of *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) are more susceptible than the 4th instar larvae to *B. bassiana* (Feng et al., 1985). In contrast, 3rd and 4th instar diamond back moth larvae are more susceptible than 2nd instar larvae to fungal pathogens (Vandenberg et al., 1998). All these show that there is no general rule regarding which developmental stage will be more susceptible (Goettel et al., 2005). However, some scientists think that the variability in larval

susceptibility to fungal infection might be linked to the molting process: insects shedding their old cuticles soon after inoculation being less susceptible, taking longer to die, or even escaping infection (Vandenberg et al., 1998).

In conclusion, this is the first study to determine the effectiveness of entomopathogenic fungi as microbial control agents against *C. arcuata*. Some of the tested isolates appear to be significant candidates for the control of this pest. In particular, *Beauveria bassiana* KTU-24 seems to be the most promising fungus considering the screening and concentration–mortality response tests. This fungus also has a significant effect on *C. ciliata*, which is another pest species belonging to Tingidae. For more proof of the effectiveness of this isolate, more studies are needed, such as horizontal transmission, field, and formulation studies.

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