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Virulence of entomopathogenic fungi against *Plagiodera versicolora* (Laicharting, 1781) (Coleoptera: Chrysomelidae)

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The willow leaf beetle, *Plagiodera versicolora* (Laicharting, 1781), is one of the most destructive pests of many *Salix* and *Populus* species worldwide. A total of 12 entomopathogenic fungi including *Beauveria bassiana* (Bals.) Vuill., *B.* cf. *bassiana*, *Metarhizium anisopliae* (Metch) Sorok, and *Isaria fumosorosea* (Wize) (formerly *Paecilomyces fumosoroseus*) were tested against the larvae of this pest under laboratory conditions in order to find any possible fungal biocontrol agent against it. A conidial concentration of 1 × 10^7 conidia ml^{-1} for each isolate was applied to third instar larvae of the pest. Among tested fungal isolates, the highest mortality was obtained as 100% from *B. bassiana* KTU-57 within 14 days after inoculation ($p < 0.05$). This isolate also caused the highest mycosis value (83%) ($p < 0.05$). It was selected for dose-response test according to the screening test, and lethal concentration at 50% (LC_{50}) value of the fungus on the larvae of the pest was calculated as 1.03 × 10^5 conidia ml^{-1} based on probit analysis. Consequently, *B. bassiana* KTU-57 appears to be a significant promising isolate against the willow leaf beetle as a possible biocontrol agent.

Key words: *Plagiodera versicolora*, *Beauveria bassiana*, lethal concentration at 50% (LC_{50}), microbial control.

INTRODUCTION

The willow leaf beetle, *Plagiodera versicolora* (Laicharting, 1781) (Coleoptera: Chrysomelidae), is one of the most important pests of many *Salix* and *Populus* species all over the world (Aslan and Ozbek, 1999; Urban, 2005). This pest is a species of the Holarctic distribution, and it occurs on extensive areas extending from Northern Africa through the whole Europe (including the European part of Russia and the Caucasus), Asia Minor, Central Asia, Siberia, China, Korea, and Japan (Brovdij, 1974; Urban, 2005). It has also wide distribution throughout Turkey (Karagoz, 1965). Both adults and larvae of the pest feed on the foliage of willow and poplar trees, leaving only the midrib and a network of veins. They emerge in May and start feeding on developing leaves. By late June, parts of heavily infested trees may appear brown, as if scorched. There may be two or three generations a year (Aslan, 2001). *P. versicolora* causes greater damages to willow and poplar trees at localities at lower altitude with higher moisture, particularly in flooded orartificially irrigated places.

In the past, many control methods have been tried to reduce damage of this pest. Beetle collection by means of sweep nets was recommended or shaking off beetles into vessels also might be useful. In addition to portable

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Table 1. The fungal isolates used in this study and their origins (Sevim et al., 2010a, b).

S/N	Species	Isolates	Locality in Turkey	Source
1	*Beauveria bassiana*	KTU-7	Yomra, Trabzon	Soil
2	*B. bassiana*	KTU-24	Samsun	*Thaumetopoea pityocampa* (Lepidoptera: Thaumetopoeidae)
3	*B. bassiana*	KTU-25	Ünye, Ordu	Soil
4	*B. bassiana*	KTU-57	Gümüşhane	*Rhynchites bacchus* (Coleoptera: Rhynchitidae)
5	*B.* cf. *bassiana*	KTU-53	Gümüşhane	Soil
6	*B.* cf. *bassiana*	KTU-55	Bayburt	Soil
7	*Metarhizium anisopliae*	KTU-2	Ardeşen, Rize	Soil
8	*M. anisopliae*	KTU-27	İkizdere, Rize	Soil
9	*M. anisopliae*	KTU-40	Akçaabat, Trabzon	Soil
10	*M. anisopliae*	KTU-51	Gümüşhane	Soil
11	*M. anisopliae*	KTU-60	Gümüşhane	Soil
12	*I. fumosorosea*	KTU-42	İkizdere, Rize	Soil

shaking off devices, a more complicated special mobile equipment was constructed. Special brushes were used for the mechanical control of larvae on small areas. In nurseries and osier plantations, beetles wintering in fallen leaves were killed by raking up and burning the leaf litter (Urban, 2005). Recently, some chemical insecticides such as Furadan G-10 and Dimiline have been utilized for controlling this pest (Jodal, 1985). However, recent concern about the hazardous effect of chemical pesticides on the environment and human has encouraged scientists to consider finding more effective and safe control agents (Sezen et al., 2004; Muratoglu et al., 2011). In the search for safer and more lasting methods, scientists have turned their attention to the possibility of using other organisms as biological control agents.

Entomopathogenic fungi are important in the natural regulation of many insect pests and pest populations are often decimated in widespread epizootics. They normally invade via the external cuticle and need not be ingested to initiate disease. This makes them prime candidates for use against plant sucking insects (Lacey and Goettel, 1995; Barta and Cagan, 2006). Some entomopathogenic fungi such as *Beauveria bassiana* (Bals.) Vuill., *Metarhizium anisopliae* (Metch) Sorok, *Isaria fumosorosea* (Wize), and *Verticillium lecanii* (Zimmerman) have been used on a commercial basis for the control of insect pests in a number of countries. From these entomopathogenic fungi, *M. anisopliae* and *B. bassiana* are the most studied species and isolates of both have been registered by The United States Environmental Protection Agency (USEPA) (Rath, 2000). Up to now, the entomopathogenic fungus, *B. bassiana*, has been extensively used for the control of many important pests of various crops around the world and it has been tested on different target insects (Campbell et al., 1985; Leathers and Gupta, 1993; Inglis et al., 1996; Ihara et al., 2001; Padmaja and Kaur, 2001; Çam et al., 2002; Susumu and Motoko, 2002; Todorova et al., 2002).

Although *P. versicolora* is an important pest species of some forest trees worldwide, studies related to controlling of the pest by entomopatohenic fungi are very limited. In this study, we tested effectiveness of 12 different entomopathogenic fungi against the third instar larvae of this pest under laboratory conditions. The result presented here can be beneficial for future biocontrol programs of this pest.

MATERIALS AND METHODS

Fungal isolates

Fungal isolates used in this study were provided from Microbiology Laboratory, Department of Biology, Karadeniz Technical University, Trabzon, Turkey. A total of 12 entomopathogenic fungi including *B. bassiana* (×4), *B.* cf. *bassiana* (×2), *M. anisopliae* (×5) and *I. fumosorosea* (×1) were used for bioassay experiments (Table 1). The fungal isolates were previously isolated from different soil and insect samples (Sevim et al., 2010a, b). They were maintained on Potato dextrose agar + 1% yeast extract (PDAY) medium (Merck).

Collection of larvae

Larvae of *P. versicolora* were collected from the infested *Salix caprea* L. forests in the vicinity of Trabzon, Turkey in August, 2011. Larvae were placed individually into plastic boxes (12 cm wide and 8 cm deep), the cover of which was perforated to permit air flow. The fresh willow leaves were provided as food until transportation to the laboratory. The collected larvae were taken to the laboratory and waited 2 to 3 days so that larvae were acclimated to the laboratory. After that, healthy third instar larvae were randomly selected and used for bioassay.

Screening test

Conidial suspensions were prepared as follows: 100 µl spore suspension of each fungal isolate (1×10^6 conidia ml^{-1}) was plated on PDAY medium and incubated at 25°C for 4 to 5 days under 12:12 photoperiod to propagate the isolates from a single spore. At the end of the incubation period, a single colony for each isolate

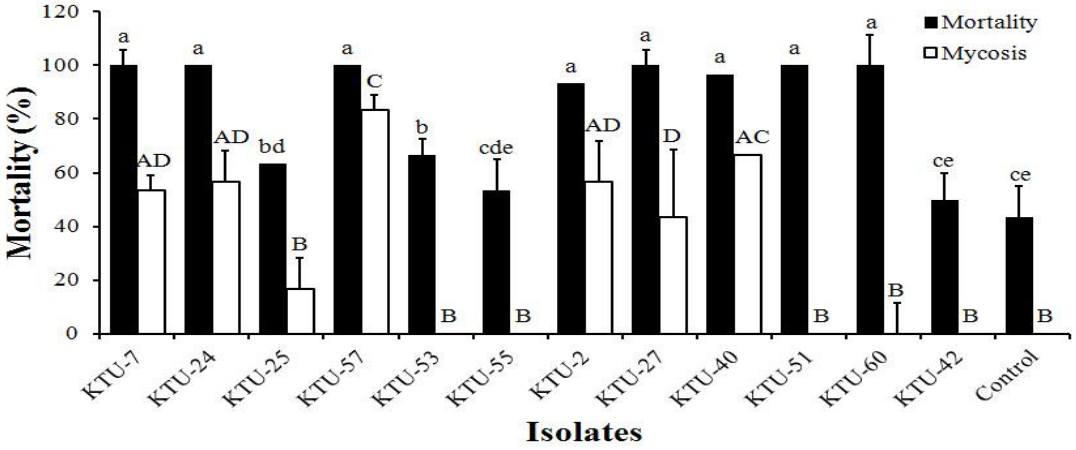

Figure 1. Mortality of the third instar larvae of *P. versicolora* within two weeks after application of the fungal isolates with concentration of 1×10^7 conidia ml^{-1}. Mortality data were corrected based on Abbott's formula (Abbott, 1925). Different upper and lower case letters show the difference between isolates according to the LSD multiple comparison test ($P < 0.05$). Bars show standard deviation. KTU-7, KTU-24, KTU-25 and KTU-57: *B. bassiana*; KTU-53 and KTU-55: *B.* cf. *bassiana*; KTU-2, KTU-27, KTU-40, KTU-51 and KTU-60: *M. anisopliae*; KTU-42: *I. fumosorosea*; Control: Tween 80 (0.01%).

was taken to another fresh PDAY medium and incubated 3 to 4 weeks under 12:12 photoperiod for sporulation. After growth period, conidia were harvested from 4-week-old cultures by adding 10 ml of sterile distilled water supplemented with 0.01% Tween 80. The conidial suspensions were filtered through two layers of sterile muslin into 50 ml plastic tube (Falkon) and then shaken for 5 min using a vortex. The concentrations of conidial suspension were adjusted to desired concentration (1×10^7 ml^{-1}) using a Neubauer haemocytometer. The viability of the conidia of the isolate was tested by plating 100 µl conidial suspensions with the concentration of 1×10^6 conidia ml^{-1}. An examination of the culture after 24 h showed that about 97% of the conidia were viable.

The third instar larvae of *P. versicolora* were fed on willow leaves at room temperature under 12:12 photoperiod until bioassays were performed. Ten larvae were used for each replicate and all tests were replicated three times. They were inoculated by dipping into conidial suspension of 1×10^7 ml^{-1} for 2 to 3 s. The control group was treated with sterile water supplemented with 0.01% Tween 80. After inoculation, all larvae were put into a plastic box (12 cm wide and 8 cm deep) including fresh willow leaves and incubated at room temperature for 2 weeks under 12:12 photoperiod. The mortalities of larvae were checked at 14^{th} day after inoculation. Dead larvae were surface-sterilized by dipping into 2% sodium hypochlorite for 3 min, followed by 70% ethanol for 3 min and washed twice in sterile distilled water. After that, they were put into the moisture chamber to stimulate fungal sporulation outside the cadaver.

Dose-response test

B. bassiana isolate KTU-57 was selected for dose-response test based on its high pathogenic effect. Healthy third instar larvae were collected from the field as described in the collection of larvae and they were randomly selected and used for dose application. They were treated with five different doses of conidial suspension of the isolate (1×10^4, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia ml^{-1}) and the control group was treated with sterile water supplemented with 0.01% Tween 80. For per replicate, 10 larvae were used and the whole experiment was repeated three times. Larvae were treated by dipping into 10 ml of each conidial suspension and then put into a plastic box (12 cm wide and 8 cm deep) together and freshly collected willow leaves were provided as the food. After that, boxes were incubated at room temperature under 12:12 photoperiod. Dead larvae were checked daily for following 12 days of inoculation.

Data analysis

Mortality values were corrected according to Abbott's formula (Abbott, 1925) and percent mycosis values were calculated. 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. Also, 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. Additionally, to determine differences among different concentrations, the data were subjected to ANOVA and later to LSD multiple comparison test. Lethal concentration at 50% (LC_{50}) value was calculated by probit analysis. All tests were carried out with SPSS 15.0 statistical software.

RESULTS

The fungal isolates caused different mortalities, and significant differences were detected among treatments ($F = 33.64$, $df = 12$, $p < 0.05$). Also, all isolates produced different mortality values in comparison to each other ($F = 32.17$, $df = 11$, $p < 0.05$). The highest mortalities were obtained from *B. bassiana* (KTU-7, KTU-24, and KTU-57) and *M. anisopliae* (KTU-27, KTU-51, and KTU-60) with 100% mortality within 2 weeks after application of fungal spores ($p < 0.05$). The other mortalities ranged from 53 to 96% (Figure 1). In the event of mycosis, all isolates caused different mycosis values ($F = 26.97$, $df = 11$, $p < 0.05$), significant differences were detected among treatments ($F = 29$, $df = 12$, $p < 0.05$). The highest mycosis value was obtained from *B. bassiana* KTU-57

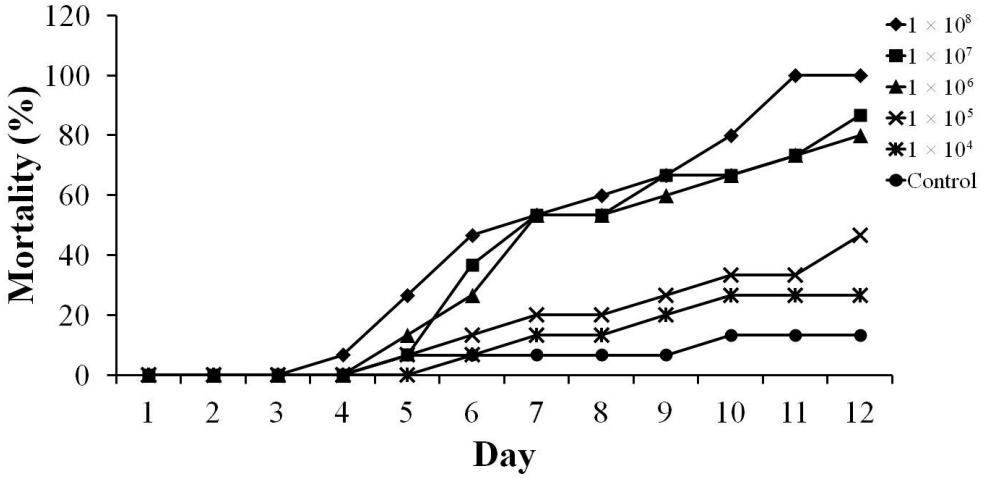

Figure 2. Cumulative mortality of the third instar larvae of *P. versicolora* after application of different doses of the fungal isolate *B. bassiana* KTU-24. Concentration unit is conidia mL^{-1}.

Table 2. Summary of probit analysis parameters from the dose-response test performed with the *B. bassiana* isolate KTU-57 against the third instar larvae of *P. versicolora*.

Bioassay	Intercept	Slope (±SE)[a]	LC_{50} (95% of fiducial limits)	$\times 2^{b}$	df
Larvae	-3.428 ± 1.457	0.684 ± 0.261	1.03×10^5 (8.38×10^2 - 9.2×10^5)	0.380	3

[a]Slope of the concentration (±standard error) response of larvae of *P. versicolora* to *B. bassiana* isolate KTU-57. [b]Pearson chi-square goodness-of-fit test on the probit model (α = 0.05).

with 83% of mycosis ($p < 0.05$). The other mycosis values of the isolates ranged from 0 to 66% (Figure 1).*B. bassiana* KTU-57 was selected for dose-response test since it caused the highest pathogenicity in the screening test. There was a significant difference among concentrations with respect to mortality ($F = 32.43$, $df = 5$, $p < 0.05$). All concentrations were different from the control group ($F = 32.43$, $df = 5$, $p < 0.05$). The mortality reached 100% within 11 days after application of the conidial concentration of 1×10^8 conidia ml^{-1} (Figure 2). Probit analysis was used to calculate LC_{50} value of the fungus. The LC_{50} of the fungus was calculated as 1.03×10^5 conidia ml^{-1} (Table 2).

DISSCUSSION

Biological control of insect pests by entomogenous fungi is very attractive, and it is alternative to chemical insecticides because they are usually safer to the environment, animals, and plants. In this study, we tested different entomopathogenic fungi belonging to order Hypocreales against *P. versicolora* under laboratory conditions in order to find more effective and safer biocontrol agent against it. We showed that the tested fungal isolates can infect the larvae of *P. versicolora*, and they were pathogenic to larvae of the pest at different ratios.

B. bassiana is a well known insect pathogenic fungus and it has been reported as a suppressive agent for several insect species worldwide (Zimmermann, 2007; Sevim et al., 2010a, b; Mukawa et al., 2011). This species is the most widely used one available commercially all over the world (Goettel et al., 2005). Products based on this species are available for use against a very wide variety of insect pests, from banana weevils [*Cosmopolites sordidus* (Germar, 1824)] in Brazil (Alves et al., 2003) to pine caterpillars (*Dendrolimus* spp.) in China (Feng et al., 1994). *B. bassiana* KTU-57 produced 100% mortality against adults of *Dendroctonus micans* (Kugel.) which is another important forest pest in the Eastern Black Sea region of Turkey (Sevim et al., 2010c). It has also good activity against adults and nymphs of *Corythucha ciliate* (Say, 1932) under laboratory conditions, 73 and 66%, respectively (unpublished data). This isolate was isolated from another coleopteran insect, *Rhynchites bacchus* (Linnaeus, 1758) in the Eastern Black Sea region of Turkey (unpublished data), so that is why it may be so pathogenic to the willow leaf beetle due to ecological compatibility of the fungus with the pest species (Maurer et al., 1997). In this study, we showed that *B. bassiana* KTU-57 produced high mortality

against the willow leaf beetle. Based on these studies, it is possible to conclude that *B. bassiana* isolate KTU-57 could be a good candidate as a possible biocontrol agent against a number of forest pests including *P. versicolora* in Turkey.

The entomopathogenic fungus *M. anisopliae* is widely used for biocontrol of pest insects, and many commercial products are on the market or under development (Zimmermann, 2007; Nishi et al., 2011). There have been many attempts to use this fungus as a practical biocontrol agent against many insect pests, from termites in USA (Copping, 2001) to grasshoppers and locusts in Africa (Lomer et al., 2001). *M. anisopliae* isolates used in this study, especially KTU-2, KTU-27 and KTU-40, also showed good activity against larvae of the willow leaf beetle. These isolates also caused high mycosis values on this pest. Among these isolates, *M. anisopliae* KTU-27 caused moderately high mortality on adults of *D. micans* (Sevim et al., 2010c). Moreover, this isolate caused 86.6% mortality against larvae of *Melolontha melolontha* (Linnaeus, 1758), which is important hazelnut and forest pest (Sevim et al., 2010a). All these studies indicate that *M. anisopliae* KTU-27 might be a good biocontrol agent against a variety of pest species in Turkey.

B. cf. *bassiana* and *I. fumosorosea* have been also isolated and used for biological control purpose from many insect species in previous studies (Gökçe and Er, 2002; Meyer et al., 2008; Sevim et al., 2010a, c). In the present study, although *B.* cf. *bassiana* and *I. fumosorosea* isolates caused significant mortality values against the willow leaf beetle, they did not produce mycosis on dead cadavers.

We also took sporulation on the host into account, since it is important determinant for fungal dissemination among pests and in the field. Therefore, isolates KTU-24 could be further investigated as biocontrol agent against *P. versicolora*, because it caused the highest mycosis on dead cadavers.

In conclusion, we tested different entomopathogenic fungi against the larvae of *P. versicolora* and showed that the fungal isolates used in this study might be utilized as a biocontrol agent against the pest. Among tested isolates, *B. bassiana* KTU-57 was the most promising one. Further studies should include mass production and efficacy of the isolate KTU-57 in the field.

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