Egyptian Journal of Biological Pest Control

RESEARCH

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Biocontrol of the western conifer seed bug, *Leptoglossus occidentalis* Heidemann (Heteroptera: Coreidae) using indigenous entomopathogenic fungi

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Abstract

Background: The western conifer seed bug, *Leptoglossus occidentalis* Heidemann (Heteroptera: Coreidae), is one of the major pest in seed orchards of various conifers, significantly affects pine seed production and causes economic damage. Biological control by natural enemies has been well studied, but its entomopathogens have been poorly studied. In this study, the efficacy of twelve indigenous entomopathogenic fungi belonging to the genera *Beauveria*, *Metarhizium*, and *Isaria* on *L. occidentalis* adults was investigated.

Results: All isolates were found to be pathogenic at a concentration of 10^7 conidia/ml where the virulence ranged from 16 to 90%. Moreover, the virulence of two *Metarhizium flavoviride* isolates (As2 and As18) reached 90% at 10^7 conidia/ml concentration. In addition, *L. occidentalis* treated with *M. flavoviride* As18 (LT_{50} = 2.53 days) died more rapidly than with *M. flavoviride* As2 (LT_{50} = 5.83 days) at the same treated concentration. For concentration-dependent virulence for isolate As18, five conidia concentrations: 10^3, 10^4, 10^5, 10^6, and 10^7 conidia/ml were used. Also, the median lethal (LC_{50}) value of As18 isolate was estimated to be 7.61×10^3 conidia/ml after 10 days of treatment. Since *L. occidentalis* is known to accumulate during overwintering, conidial transmission among adults was investigated. Unfortunately, horizontal transmission between adults did not occur.

Conclusion: The isolate *M. flavoviride* As18, which had low LC_{50} and LT_{50} values, appeared to be a promising biological control agent against *L. occidentalis*. This isolate should be formulated as a myco-insecticide and tested under field conditions in further studies.

Keywords: *Leptoglossus occidentalis*, *Metarhizium flavoviride*, Virulence, Horizontal transmission, Biocontrol

Background

The western conifer seed bug, *Leptoglossus occidentalis* Heidemann (Heteroptera: Coreidae) is an invader pest of coniferous trees. It is native to North America (Allen 1969) and spread from the central to the eastern regions since the mid-nineteenth century. In Europe, it was reported for the first time in Italy (1999) and recorded all over Europe at the beginning of the twenty-first century. It also reached China (Zhu 2010), Russia (Gapon 2013), Japan (Ishikawa and Kikuhara 2009), Turkey (Fent and Kment 2011), Tunisia (Ben Jamâa et al. 2013).

The pest is a sucking insect that feeds on cones and seeds of at least 30 conifer species (Farinha et al. 2021). The reported damages caused by the insect include massive abortion of conelets and a high percentage of empty seeds in ripened cones, a phenomenon known as Dry Cone Syndrome (El Khoury et al. 2021). Increasing populations of the pest have led to a 95% decline in pine nut

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production in Mediterranean countries (Parlak 2017). In addition, *L. occidentalis* is a vector of *Diplodia sapinea* spores, the fungus responsible for Diplodia tip blight of pine trees.

Generally, broad-spectrum insecticides have been used to control the pest. However, their usage is limited due to their toxic effects on bees and beneficial insects. To overcome this limitation, the use of entomopathogenic fungi (EPF) is a good alternative tool (Sonmez et al. 2022). Many studies covered the insecticidal activity of EPF (Biryol et al. 2022). About the use of fungi for the control of *L. occidentalis*, a preliminary study by Rumine and Barzanti (2008) showed that isolates of *Beauveria bassiana* were the most pathogenic to adults of *L. occidentalis*. In another study, Barta (2010) evaluated the virulence of *B. bassiana*, *Isaria fumosorosea*, and *Metarhizium anisopliae* against this pest under laboratory conditions and reported that isolates of *I. fumosorosea* were the most virulent. However, studies on the control of *L. occidentalis* with EPF are limited; therefore, the objective of the present study was to test the efficacy of 12 indigenous EPF against *L. occidentalis* and to determine the concentration response and horizontal transmission ability of the most effective isolate.

Methods

Insects

Leptoglossus occidentalis adults were collected using landing nets in Bursa, Turkey. The collected individuals were placed in rearing cages ($50 \times 40 \times 40$ cm) with perforated lids. Insects were fed on fresh immature pine cones at 20 ± 2 °C, 70 ± 10% relative humidity (RH) and under a natural photoperiod until bioassays were performed (Barta 2010).

Entomopathogenic fungi

The fungi used in the study were obtained from the microbiology laboratory of the Department of Biology, Karadeniz Technical University. Twelve EPFs belonging to the genera *Beauveria*, *Metarhizium* and *Isaria* were used in the study (Table 1). A loopful of spore suspension from glycerol stock culture was inoculated onto Potato Dextrose Agar (PDA) plates and incubated for 3 days at 28 ± 2 °C in the dark. Then, a single colony was transferred to a fresh PDA plate and incubated for 2 weeks until the plates were fully grown (Biryol et al. 2021a).

Preparation of conidia suspensions

Conidial suspensions were prepared from 2-week-old cultures. Fungal spores were collected from Petri dishes using a sterile scalpel and added to 10 ml of sterile distilled water containing 0.01% (v/v) Tween 80. The conidial suspensions were filtered through several layers of sterile muslin to remove fungal debris after the suspension was homogenized with a vortex for 2 min. Filtrates containing conidia were examined under a light microscope, using a hemocytometer, and conidia in the suspensions were quantified by direct counting. The concentration was adjusted to 10^7 conidia/ml for use in the bioassay (Biryol et al. 2021a).

Table 1 Entomopathogenic fungi tested on *Leptoglossus occidentalis* and their origin

Fungi	Isolate	Origin	References
Isaria fumosorosea	KTU-1	Soil	Sevim et al. (2010a)
	KTU-42	Soil	Sevim et al. (2010a)
Beauveria bassiana	KTU-24	*Thaumetopea pityocampa* (Lepidoptera: Notodontidae)	Sevim et al. (2010b)
	K4	*Hypera postica* (Coleoptera: Curculionidae)	Yucel et al. (2018)
	K5	*H. postica*	Yucel et al. (2018)
	HP4	*H. postica*	Yucel et al. (2018)
	HP5	*H. postica*	Yucel et al. (2018)
	Pa4	*Pristiphora abietina* (Hymenoptera: Tenthredinidae)	Biryol et al. (2021a)
	Rh	*Rhynchites bacchus* (Coleoptera: Rhynchitidae)	Sevim et al. (2014)
Metarhizium flavoviride	As2	*Amphimallon solstitiale* (Coleoptera: Scarabaeidae)	Biryol et al. (2020)
	As18	*A. solstitiale*	Biryol et al. (2020)
Metarhizium brunneum	Gg12	*Gryllotalpa grylotalpa* (Orthoptera: Gryllotalpidae)	Sonmez et al. (2022)

Screening experiments

The efficacy of the fungi was tested on adults of *L. occidentalis*. After applying 5 ml (10^7 conidia/ml) of the fungal suspensions to the adults with a hand-operated sprayer, the insects were placed in a rearing cage ($50 \times 40 \times 40$ cm) containing fresh pine shoots. Thirty adults were used for each treatment and repeated three times. Control adults were treated with 0.01% aqueous Tween80. Bioassays were performed under laboratory conditions at 20 ± 2 °C, 70 ± 10% RH and at a natural photoperiod. Dead insects were transferred in a humidity chamber after surface sterilization with 70% alcohol to stimulate fungal sporulation. Mortality and mycosis were recorded daily for 10 days.

Concentration response experiment

For concentration-dependent virulence tests, the *M. flavoviride* As18 isolate with a mortality rate of 90% and a relatively short LT_{50} was used at five conidia concentrations: 10^3, 10^4, 10^5, 10^6, and 10^7 conidia/ml. *L. occidentalis* adults were sprayed using the same procedure as described above. Control adults were treated with an aqueous solution of 0.01% Tween 80. Three replicates of 30 larvae were used for each concentration. Treated adults were maintained as previously described, and each insect was monitored daily for 10 days after treatment.

Horizontal transmission experiment

Conidial transmission of As18 isolate among *L. occidentalis* was performed on overwintering adults under laboratory conditions. The experiment was performed as previously described by Biryol et al. (2021a) with a concentration of 1×10^7 conidia/ml. Horizontal transmission was studied with fungus-treated and untreated adults at four vector ratios of 0, 25, 50, 75, and 100% in groups of 40 insects with three replicates. After fungus-treated individuals were air-dried at room temperature, both treated and untreated individuals were released into the rearing cage. After 10 days, mortality and mycosis were assessed as described above.

Data analysis

Mortality and mycosis rates were corrected for control mortality using the Abbott formula (Abbott 1925). Data were analyzed using ANOVA and then the least significant difference (LSD) test to compare test isolates with each other and with the control group ($p < 0.01$). The lethal time value required to kill 50% (LT_{50}) of *L. occidentalis* exposed to the fungal isolates and the median lethal concentration (LC_{50}) of the most toxic isolate were estimated with 95% confidence interval by probit analysis. Kaplan–Meier survival analysis was also performed, followed by a log-rank test for multiple comparisons to compare different survival curves. The SPSS Statistics 25 software package was used for the analyses (SPSS Inc. Chicago, IL, USA).

Results

Screening and concentration response experiments

Screening tests were performed on *L. occidentalis* adults with 12 EPF whose insecticidal activity on various insects had been determined in previous studies. In screening tests, which were carried out at the concentration of 10^7 conidia/ml, all isolates were found to be pathogenic. However, a highly significant difference in mortality of *L. occidentalis* was observed among isolates ($F = 92.2$; $df = 11$ $p < 0.01$). Mortality rates ranged from 16 to 90%. Moreover, two of the *M. flavoviride* (As2 and As18) isolates caused 90% mortality (Fig. 1). In addition, *L. occidentalis* treated with *M. flavoviride* As18 died more rapidly than with *M. flavoviride* As2 at a conidial concentration of 10^7 conidia/ml. LT_{50} values were 5.83 and 2.53 days for isolates As2 and As18, respectively, at same concentration (Table 2). On the other hand, the lowest mortality rate was observed in the insects treated with *B. bassiana* KTU-24 (16%) and *I. fumosorosea* KTU-1 (26%). Mortality rate in the control group was less than 5%. Dead insects showed symptoms of *M. flavoviride* infection after incubation in the humid chamber (Fig. 2).

Metarhizium flavoviride As18 isolate with 90% mortality rate and a relatively short LT_{50} was selected for the concentration–response experiment. Survival analysis showed that there was a significant difference among the concentrations (log-rank, $p < 0.01$). However, there was no statistical difference between the concentrations of 10^4 and 10^5 conidia/ml (log-rank, $p > 0.01$) (Fig. 3).

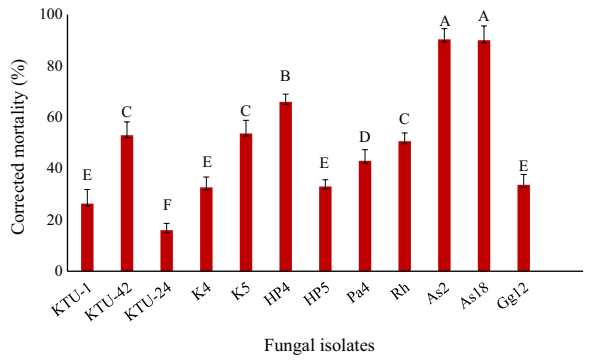

Fig. 1 Screening test of entomopathogenic fungi on adults of the pest under laboratory condition at the concentration of 10^7 conidia/ml. Different case letters represent statistically significant differences among mortalities according to the least significant difference (LSD) multiple comparison test ($p < 0.01$). Mortality indicates the mean of three replications. Bars show standard error

Table 2 Median lethal time (LT_{50}) of fungal isolates against the adult of *Leptoglossus occidentalis* at concentration of 10^7 conidia/ml

Fungal isolates	LT_{50} (FL, 95%) (days)	Slope ± SE	*df*	χ^2
KTU-1	14.4 (11–27.4)	2.08 ± 0.32	8	19.4
KTU-42	9.82 (9.16–10.7)	4.02 ± 0.41	8	5.25
KTU-24	35 (22–88)	1.2 ± 0.29	8	7.14
K4	12.4 (10.9–15.0)	2.62 ± 0.35	8	9.54
K5	9.16 (8.5–10.0)	3.4 ± 0.31	8	5.05
HP4	8.38 (7.88–9.01)	3.68 ± 0.32	8	9.63
HP5	12.55 (11.22–14.94)	3.78 ± 0.55	8	4.25
Pa4	11 (9.9–12.84)	2.94 ± 0.31	8	5.21
Rh	12.79 (11.34–15.39)	3.6 ± 0.47	8	3.26
As2	5.83 (4.31–5.71)	3.28 ± 0.29	8	45.2
As18	2.73 (2.12–2.98)	1.81 ± 0.14	8	8.32
Gg12	9.17 (8.53–10)	3.53 ± 0.32	8	4.74

FL fiducial limit, *SE* standard error, *df*: degree of freedom, χ^2 Chi square

The LC_{50} value of *M. flavoviride* As18 for *L. occidentalis* was estimated to be 7.61×10^3 conidia/ml using probit analysis.

Horizontal transmission experiments

Conidial transmission from treated to untreated adult bugs over a 10-day period was assessed and As18 isolate showed no ability to disseminate. None of the insects were died in the non-treated control groups during experiments. Mortality rate was 22.7% at vector ratio 25%. A similar trend was observed at vector ratios 50 and 75% with 43.8 and 70% mortality, respectively. When 100% of adult beetles were treated with 10^7 conidia/ml concentration of As18, 95.5% mortality was observed (Table 3).

Discussion

Entomopathogenic fungi (EPF) have long been associated with different families in heteropteran species. In the present study, 12 isolates of three EPFs (*B. bassiana*, *M. flavoviride* and *M. brunneum*) were examined under laboratory conditions for their virulence to adult of *L. occidentalis*. Isolates of all three hypocrealean fungi were pathogenic to adults of *L. occidentalis*, although their virulence varied greatly among isolates. Isolates of *M. flavoviride* (As2 and As18) were more virulent than isolates of the other two fungal species. On the other hand, a preliminary study by Rumine and Barzanti (2008) showed that isolates of *B. bassiana* were most pathogenic to adults of *L. occidentalis*. In another study, Barta (2010) evaluated the virulence of *B. bassiana*, *I. fumosorosea*, and *M. anisopliae* to this pest under laboratory conditions and reported that isolates of *I. fumosorosea* were more virulent. Previous studies have shown that the difference in virulence of EPF is related to a higher germination rate, hyphal growth rate and production of cuticle-degrading enzymes because infection can potentially occur much more quickly (Zhang et al. 2011). In the present study, although *M. flavoviride* As2 and As18 isolates caused 90% mortality at 10^7 conidia/ml concentration in screening experiment, the LT_{50} values were 5.83 and 2.53 days at 10^7 conidia/ml concentration, respectively. Therefore, the concentration–response experiment was conducted with As18 isolate, which had the lowest LT_{50} value at the concentration of 10^7 conidia/ml. The LC_{50} value was estimated as 7.6×10^3 conidia/ml after 10 days of treatment for adults of *L. occidentalis*. Barta (2010) isolated two EPF, *I. fumosorosea* (AMSAS 06) and *B. bassiana* (AMSAS 03), from dead *L. occidentalis* and determined LC_{50} values as 0.86×10^5 and 10.3×10^5 conidia/ml, respectively. Also, they reported that soil originated *B.*

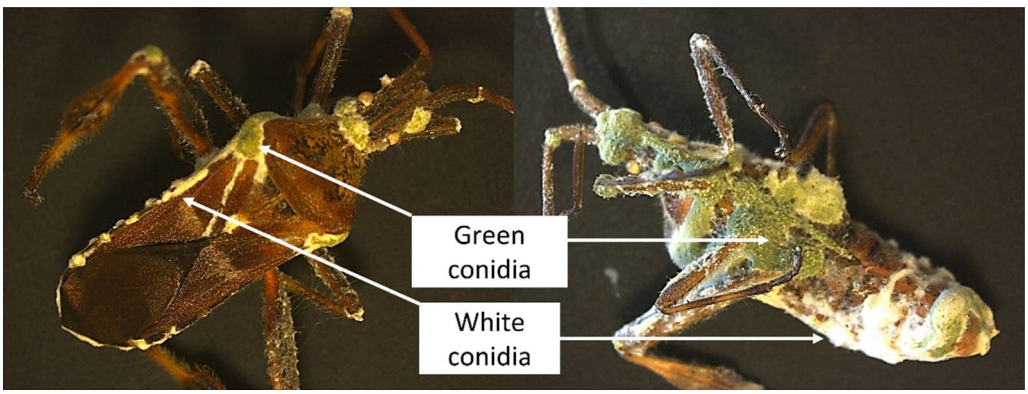

Fig. 2 Mycosis of the pest exposed to isolate of *Metarhizium flavoviride* As18. The white conidia were initially observed on the insect cadaver 6 days after treatment in the moist chamber, then green conidia were observed 10 days after treatment

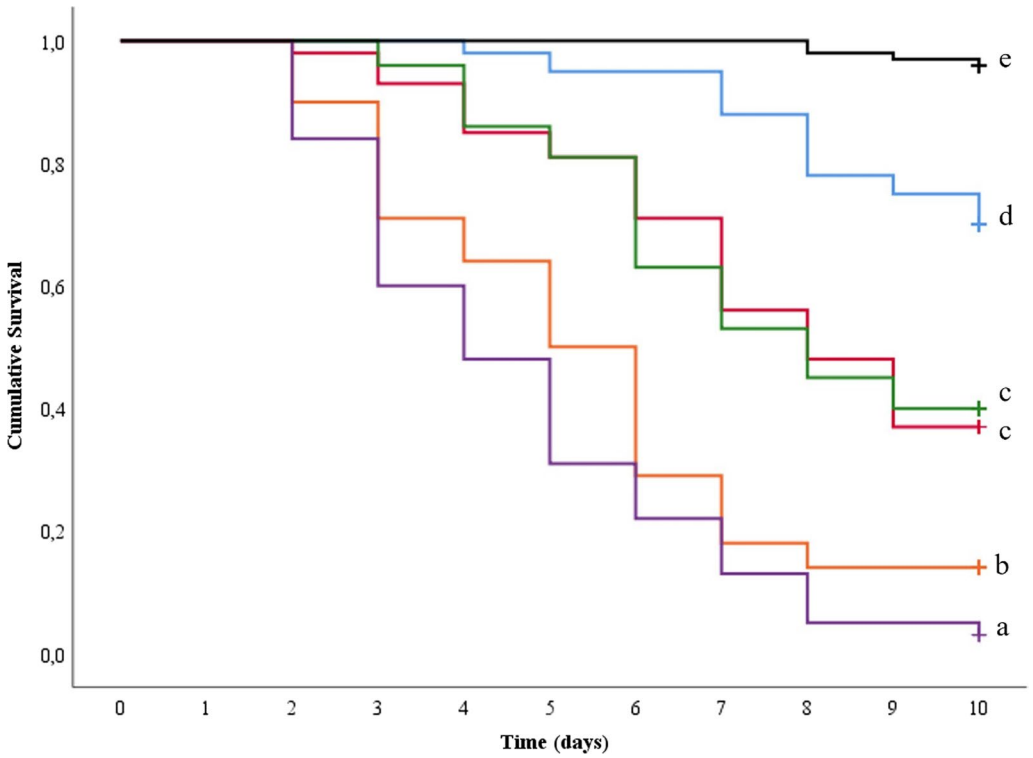

Fig. 3 Kaplan–Meier survival diagram for adult of *Leptoglossus occidentalis* exposed to isolate *Metarhizium flavoviride* As18 at five different concentrations. Holm-Sidak estimates of survival data show significant differences between treatment groups represented by lowercase letters

Table 3 Horizontal transmission of fungal conidia of *Metarhizium flavoviride* (As18) from treated *Leptoglossus occidentalis* adults to fungus-free adults at five infection rates (%) 10 days after treatment

Vector ratio (%)	Mortality (%)
0	1.66
25	22.7
50	43.8
75	70.0
100	95.5

Total number of adults was 40; 0% vector ratio involved non-treated control insects, 25% vector ratio involved 10 fungi treated adults and 30 non-treated adults, 50% vector ratio involved 20 fungi treated adults and 20 non-treated adults, 75% vector ratio involved 30 fungus-treated adults and 10 non-treated adults and 100% vector ratio involved 40 fungus-treated adults. Mortality indicates the mean of three replications

bassiana (SUA a38) isolate was less virulent and LC_{50} value was 8.46×10^6 conidia/ml. Generally, isolates obtained from naturally infected insects are more virulent than those isolated from different habitats (Biryol et al. 2020). As in this study, EPF isolated from a particular host or soil have shown to be virulent to other hosts. Biryol et al. (2021b) reported that soil originated *M. anisopliae* (KTU-51) isolate showed 100% mortality on *Orosanga japonica* Melichar (Hemiptera: Ricaniidae).

Since *L. occidentalis* is known to accumulate for overwintering in many different places, e.g., under loose bark, in holes of dead logs, or in bird nests, conidial transmission among adults may be possible during this time. The tested isolate As18 had not transmitted horizontally within a time. This is the first report of horizontal transmission of EPF among the *L. occidentalis* population. A similar study to ours, found that nymph mortality of *Cimex lectularius* L. (Hemiptera: Cimicidae) induced by horizontal transfer was low using *B. bassiana* and did not reach more than 11%, even the ratio of 67%. Horizontal transmission of conidia in aggregations may have been limited by an increase in anti-fungal secretions of insects (Ulrich et al. 2015). In addition, some studies have emphasized the importance of mating activity for horizontal transmission (Srei et al. 2020). Because our study was conducted on overwintering adults, mating did not occur as well horizontal transmission.

Conclusions

The virulence of twelve EPF belonging to the genera *Beauveria*, *Metarhizium* and *Isaria* was evaluated on the adults of *L. occidentalis*. *M. flavoviride* As18 was determined to be the most virulent and fast killing isolate for microbial control of *L. occidentalis*. This is the first study to investigate the efficacy of *M. flavoviride* on *L. occidentalis* adults. Based on the results of the present study, *M. flavoviride* isolate As18 could prove to be a suitable microbial control agent that could be used as part of an IPM strategy of *L. occidentalis*. Further studies should be conducted to validate these results under field conditions.

Acknowledgements
Not applicable.

Author contributions
ID and SB designed the study. AE and OA performed the study. ID and AE took part in writing original draft. SB and OA involved in review and editing. All authors read and approved the final manuscript.

Funding
Not applicable.

Availability of data and materials
All data generated and analyzed during this study are indicated in the manuscript.

Declarations

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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Received: 14 August 2022 Accepted: 6 December 2022
Published online: 10 December 2022

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