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Preliminary evaluation of insect-pathogenic Hypocreales against *Leptoglossus occidentalis* (Heteroptera: Coreidae) in laboratory conditions

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Abstract

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Leptoglossus occidentalis, a species native to North America, is considered a major pest of conifer seed orchards in its natural area of distribution. Recently, the seed bug was accidentally introduced into southern Europe and its populations have been expanding throughout Europe. In the course of population study of this seed bug in Slovakia, two entomopathogenic fungi were identified from dead individuals, *Isaria fumosorosea* and *Beauveria bassiana*. In the present study, we evaluated pathogenicity of six indigenous isolates of three entomopathogenic fungi, *B. bassiana*, *I. fumosorosea* and *Metarhizium anisopliae*, to adults of the exotic coreid bug under laboratory conditions. All the isolates were virulent to the seed bug, but pathogenicity varied significantly among the isolates. Generally, isolates obtained from naturally infected *L. occidentalis* were more virulent than those isolated from soil samples. The LC_{50} values, as estimated by probit analysis, ranged from 0.86 to 84.68 × 10^5 conidia/ml and *I. fumosorosea* isolates reached the lowest median lethal concentrations. The results of this bioassay showed that *I. fumosorosea* a has potential as a microbial control agent of *L. occidentalis*.

Key words

Hypocreales, natural enemies, virulence, western conifer seed bug

Introduction

Leptoglossus occidentalis Heidemann (Heteroptera: Coreidae), the western conifer seed bug, is a species native to North America, is considered a major pest of conifer seed orchards (McPherson et al., 1990). This seed bug was first described from California in 1910 (Bernardinelli and Zandigiacomo, 2001) and since the second half of the last century its populations have been expanding eastward from its natural habitat on the west coast of North America (McPherson et al., 1990; Wheeler, 1992). In 1999, it was recorded from Europe, near the town of Vicenza in northern Italy, for the first time (Tescari, 2001). This first European record was soon followed by finds in further localities in Italy and other countries throughout Europe (e.g. Bernardinelli and Zandigiacomo, 2001, 2002; Gogala, 2003; Moullet, 2006; Aukema and Libeer, 2007; Lis et al., 2008). In Slovakia, the occurrence of the western conifer seed bug was studied in collections of conifers in the Arboretum Mlyňany SAS as well as in parks and public greenery of several settlements in south-western Slovakia during the summer 2008. During the survey, the seed bug was recorded feeding on 18 conifer species (Barta, 2009). In Slovakia, an appearance of natural enemies in population of this exotic species was also studied and two entomopathogenic fungi were identified from collected individuals; they were *Isaria fumosorosea* Wize and *Beauveria bassiana* (Balsamo) Vuillemin (Barta, 2009). In the literature, there is only limited information about activity of the entomopathogenic fungi in the populations of the seed bug. The

Slovak findings are probably the first records of natural infection of *L. occidentalis* by these fungi. However, in laboratory conditions this seed bug showed a susceptibility to an artificial inoculation with *B. bassiana* (Rumine and Barzanti, 2008).

The hypocrealean entomopathogenic fungi are ubiquitous organisms attacking various arthropods by causing acute mycoses. They can spread fast among insect populations horizontally via aerially produced conidia and infect its host by penetration of the cuticle with germ hyphae. After crossing the insect integument, the fungi grow within the internal fluids, sponging degraded proteins and fat bodies, and produce toxins which kill the host. After the host's death, the mycelium grows throughout the cadaver and protrudes outside completing the life cycle by rich conidial sporulation (Hajek and St Leger, 1994). Many strains of entomopathogenic fungi have been isolated and tested on different pests in a variety of cropping systems (e.g. Legaspi et al., 2000; Leland et al., 2005; Pu et al., 2005; Liu and Bauer, 2008). Selected strains have been successfully licensed for commercial use against whiteflies, aphids, thrips and numerous other insect pests in recent years (Shah and Pell, 2003). However, until now, the development of microbial control agents for *L. occidentalis* attracted only little attention (Rumine and Barzanti, 2008).

The main objective of this study was to evaluate the pathogenicity of indigenous Slovak isolates of hypocrealean insect-pathogenic fungi against the exotic coreid bug, *L. occidentalis*. Under laboratory conditions, we determined the susceptibility of adults to six fungal isolates belonging to three fungal species.

Material and methods

Insects

Adult individuals of *L. occidentalis* used in the bioassay were collected by sweep netting in the Arboretum Mlyňany SAS (48°19'12" N, 18°22'09" E) in Slovakia. All collected individuals were placed in a rearing cage (300 × 300 × 400 mm covered with fine nylon fabric) in the laboratory at 20 ± 2°C and 70 ± 10% relative humidity (RH) with a natural photoperiod. They were kept in the cage until their use in the bioassay (usually no longer than 48 h) and provided with fresh food (Douglas-fir cones).

Fungal isolates

The origin and hosts of 3 isolates of *B. bassiana*, 2 isolates of *I. fumosorosea* and 1 isolate of *M. anisopliae* used in this study are given in Table 1. The fungi were cultivated on Sabouraud-dextrose agar (SDA) in Petri dishes and incubated at 25 ± 2°C with a 16/8 (L/D) photoperiod. Aerial conidia were harvested from 15-day-old cultures and suspended in 100 ml of sterile distilled

Table 1. Fungal isolates assayed against adults of *L. occidentalis*

Beauveria bassiana		
	Isolate	SUA a38
	Host	*Galleria mellonella* (L.) (Lepidoptera: Pyralidae) as bait from soil
	Site and date of origin	Slovakia (48°17'55.86" N, 19° 3'13.54" E), 2008
	Isolate	SUA b38
	Host	*G. mellonella* (L.) (Lepidoptera: Pyralidae) as bait from soil
	Site and date of origin	Slovakia (48°07'43.62" N, 17°47''03.85" E), 2008
	Isolate	AMSAS 03
	Host	*L. occidentalis* Heidemann (Heteroptera: Coreidae)
	Site and date of origin	Slovakia (48°19'12.66" N, 18°22'08.51" E), 2009
Isaria fumosorosea		
	Isolate	SUA f84
	Host	*G. mellonella* (L.) (Lepidoptera: Pyralidae) as bait from soil
	Site and date of origin	Slovakia (48°17'55.86" N, 19°33'13.54" E), 2008
	Isolate	AMSAS 06
	Host	*L. occidentalis* Heidemann (Heteroptera: Coreidae)
	Site and date of origin	Slovakia (48°19'12.66" N, 18°22'08.51" E), 2009
Metarhizium anisopliae		
	Isolate	SUA d26A
	Host	*G. mellonella* (L.) (Lepidoptera: Pyralidae) as bait from soil
	Site and date of origin	Slovakia (48°17'29.55" N, 18°07'20.80" E), 2008

water with 0.05% (v/v) Tween 80 (Sigma-Aldrich, India). The conidial suspensions were filtered through several layers of cheesecloth to remove mycelial mats. Conidial concentrations were adjusted to 1×10^8 conidia ml^{-1} (stock suspensions). Conidia in the suspensions were quantified by direct counting with an optical microscope using an improved Neubauer chamber. Viability of conidia was assessed before preparing of final suspensions in germinating tests. The stock suspensions (0.5 ml) for each isolate were pipetted on an SDA plate and incubated at 20 °C. After 24 h the rate of conidial germination was determined by counting 100 conidia in four different fields of view (400 spores per plate, magnification = 500×). The conidia were categorised into two groups: viable conidia identified by production of germ tubes, and non-germinating conidia. Only conidia with a germ tube longer than its width were considered germinated. Only fungal cultures in which more than 90% of the conidia germinated used in the bioassay.

Laboratory bioassay

For all test isolates, five aqueous suspensions were prepared from the stock in a logarithmic series from 1×10^8 to 1×10^4 conidia ml^{-1} in Tween 80 (0.05%, v/v). The concentrations were determined based on pre-tests, in which a concentration that would kill about 10% and another that would kill 90% of treated insects was identified. The other concentrations used were distributed between these extremes. For each concentration, a group of 20 *L. occidentalis* adults were treated by direct immersion in the conidial suspension for 10 s. A further 40 adults were immersed in 0.05% Tween 80 (v/v) alone as controls. The treated and control insects were incubated in groups of 20 in transparent polypropylene boxes (500 ml) for a period of 10 days at 23 ± 2°C, saturated RH and with a natural photoperiod. The test insects were observed at 24-h intervals to record mortality and fresh food (a Douglas-fir cone) was changed at 2-day intervals. All dead individuals were surface sterilised in a sodium hypochlorite solution (1%, w/v) for 30 s, rinsed twice in sterile distilled water and incubated individually in Petri dishes containing water agar (2%, w/v) for 7 days to stimulate development of mycosis and confirm infection by the test fungi. The bioassay was repeated 3 times at intervals of 1 week for all isolates.

Statistical analysis

Cumulative percentage mortality data from the bioassay (10 days after treatment) were corrected for natural mortality using Abbott's formula (Abbott, 1925) and analysed with the Probit analysis (Finney, 1971) in Minitab 14® (© 2004 Minitab Inc.) to estimate LC_{50} for each isolate. Analysis of variance (Anova) was used to determine the significant differences between the treatments. Tukey's HSD multiple comparison followed if significant differences were detected.

Results

Three isolates of *B. bassiana*, two isolates of *I. fumosorosea* and one isolate of *M. anisopliae* were screened for their virulence to adults of *L. occidentalis* in the laboratory. The basic measure of virulence generated in this study was the median lethal concentration (LC_{50}) expressed as conidia ml^{-1} of test suspension and based on mortality recorded 10 days post-inoculation. Our results indicate that the western conifer seed bug adults are sensitive to isolates of all the three hypocrealean fungi tested. In general, the percentage mortality of experimental insects increased with the concentration of conidia in the suspensions what allowed to estimate a median lethal concentration. Figure 1 shows the percentage mortality caused by the fungal isolates at different rates of conidial concentration. LC_{50} values for adult *L. occidentalis* are presented in Table 2. High inter-specific variability was recorded in virulence of test isolates and significant differences occurred among them ($F_{5.06} = 49.39$, $P < 0.01$). The LC_{50} values, as estimated by probit analysis, ranged from 0.86 to 84.68 $\times 10^5$ conidia ml^{-1}. The mean spore viability of the test isolates was 91.00 ± 0.70% – 97.00 ± 0.91% (Table 2) during laboratory bioassays with significant differences among the five test isolates ($F_{4.25} = 5.47$, $P < 0.01$). However, no significant relationship was observed between conidial viability and median lethal concentration of the five isolates ($R^2 = –0.186$, $P = 0.72$). Mortality in the control groups ranged from 0 to 10% ($\bar{x}$ = 3.06 ± 0.90%, $n = 18$). The lowest LC_{50} values were obtained for *I. fumosorosea* isolates and the estimated LC_{50} for these isolates were significantly lower ($P < 0.05$), when compared with the remaining isolates. The least virulent isolate was that of *B. bassiana* species, isolate SUA a38. Virulence of *B. bassiana* isolates ranged from 10.35 to 84.68 $\times 10^5$ conidia ml^{-1} and high intra-specific variation was observed. The intra-specific difference among *B. bassiana* isolates was statistically significant ($P < 0.05$), however significant variability was not detected between *I. fumosorosea* isolates ($P > 0.05$). Generally, isolates obtained from naturally infected *L. occidentalis* (AMSAS 03 and AMSAS 06) were more virulent than those of the same fungal species but isolated from soil samples. Probit regression slopes in these assays varied from 0.20 to 0.50.

Discussion

The three fungal species tested in the present study are considered facultative insect pathogens (Bidochka et al., 2002; Cory and Ericsson, 2010) and we successfully

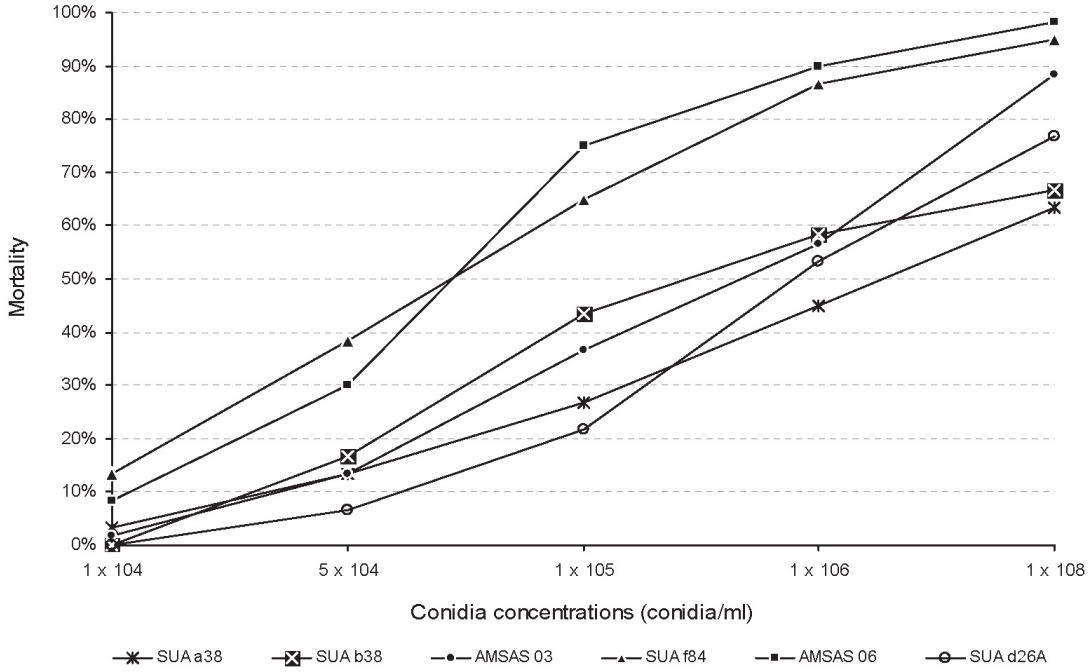

Fig. 1. Mean percentage mortality of *L. occidentalis* adults at different levels of conidia concentration 10 days after exposure to the six test isolates of entomopathogenic fungi in the laboratory bioassays.

Table 2. Probit analysis results for test isolates against adults of *L. occidentalis* evaluated 10 days after exposure to conidial suspensions

Isolate	LC_{50} (x 105)[a]	95% fiducial CI (x 105)[a]	Slope ± SE	X^{2b}	P	Conidial viability
Beauveria bassiana						
SUA a38	84.68 d[c]	33.41–306.57	0.20 ± 0.03	2.38	0.000	91.50 ± 0.65% ab[c]
SUA b38	27.52 b	11.98–79.73	0.20 ± 0.02	2.08	0.000	97.00 ± 0.91% d
AMSAS 03	10.35 a	5.85–19.98	0.31 ± 0.03	3.60	0.000	92.50 ± 1.19% ab
Isaria fumosorosea						
SUA f84	1.08 a	0.63–1.83	0.34 ± 0.04	1.99	0.000	95.50 ± 1.44% cd
AMSAS 06	0.86 a	0.59–1.25	0.50 ± 0.06	3.36	0.000	91.00 ± 0.70% a
Metarhizium anisopliae						
SUA d26A	47.13 c	23.38–108.13	0.30 ± 0.03	3.61	0.000	94.00 ± 0.91% bc

[a]Values of median lethal concentration and 95% fiducial confidence intervals are expressed in conidia per millilitre.
[b]Pearson chi-square goodness-of-fit test on the probit model ($\alpha = 0.05$, df = 3).
[c]Values followed by the same letter in the column are not significantly different (95% Tukey's HSD test).

demonstrated their pathogenicity to the coreid bug, *L. occidentalis*. In the laboratory assays, we showed that adults of the western conifer seed bug are susceptible to all the test fungal species, although virulence varied greatly among the isolates. Based on data presented in this study, adults of *L. occidentalis* are significantly more susceptible to the isolates of *I. fumosorosea* than to the isolates of remaining two fungal species. The results showed that *I. fumosorosea* has a good potential to be considered a possible biological control agent of *L. occidentalis*. The use of entomopathogenic fungi in a pest management is not a new idea. The three fungal species tested in this bioassay are extensively studied. They are the most commonly used fungi for control of insect pests and form the basis of a number of commercially available pesticides (Shah and Pell, 2003). However, we did not find any studies determining pathogenic activity of the entomopathogenic fungi against *L. occidentalis* in the literature, except the preliminary results presented by Italian authors (Rumine and Barzanti, 2008). In the Italian bioassay, *B. bassiana* successfully manifested virulence to *L. occidentalis* under

laboratory conditions. Several other coreid pest species were also tested for their susceptibility to entomopathogenic fungi. For instance, in Nicaragua, pathogenicity of *B. bassiana* and *M. anisopliae* was assessed against adults of another *Leptoglossus* species, *L. zonatus* Dallas, and application of these isolates was effective both in the laboratory and in field conditions (Grimm and Guharay, 1998). *B. bassiana* or *M. anisopliae* also successfully infected adults of other coreid bugs, *Riptortus linearis* (Fabricius) with LC_{50} of 1.1×10^6 conidia ml^{-1} (Hu et al., 1996), *Paradasynus rostratus* (Distant) (Mohan et al., 2001) and *Clavigralla tomentosicollis* Stål with LC_{50} ranging from 9.8×10^4 to 1.8×10^5 (Ekesi, 1999). According to data presented by the above authors, the hypocrealean entomopathogens have potential for their use in alternative control strategies of coreid bugs.

As observed in our bioassay, mortality of test insects increased with conidial concentration. Similar relationship is commonly observed in other dose-mortality studies (e.g. Hu et al., 1996; Ekesi, 1999; Eken et al., 2006; Rahman et al., 2010). We found out that conidial viability did not directly correlate with fungal virulence what was also observed for *B. bassiana* when tested against *Lygus lineolaris* (Palisot de Beauvois) under laboratory conditions (Liu et al., 2003). Mortalities in the control groups were low ($\bar{x}$ = 3.06%) in our bioassays confirming that the corrected mortalities obtained in the treatments were due to the pathogenicity of the entomopathogens rather than to other factors.

It is generally admitted that the most virulent fungal isolates are the ones isolated from the host (Papierok et al., 1984). This is true in many cases as some reported in works by Peña et al. (1996), Ekesi (1999), or Santoro et al. (2008), where fungal isolates that originated from particular insect species were more virulent to this species than those isolates obtained from other hosts. This was also the case in our study, where the most virulent isolates did originate from *L. occidentalis*.

In summary, these initial laboratory bioassays identified *I. fumosorosea* as a possible biological control agent of *L. occidentalis*. However, susceptibility of insects to entomopathogens demonstrated in laboratory usually do not relate to infection rate obtained in fields what pointing out differences between physiological and ecological susceptibility of insects. Therefore, further research is needed to verify *L. occidentalis* susceptibility in forest environment. As a matter of fact susceptibility relates to the physiology and behaviour of insect, which may encourage or discourage infection process. Moreover, since the entomopathogenic fungi are transmitted horizontally in the environment, they are depended considerably upon environmental conditions. Environmental factors may thus directly or indirectly influence host and pathogen populations as well as a means of inoculum transmission.

To our knowledge, this study is the first that demonstrate successful artificial inoculation of *L. occidentalis* with *I. fumosorosea* and *M. anisopliae*. According to our results, indigenous entomopathogenic mycoflora proved their capability of invading and killing this exotic coreid bug in the laboratory trials. However, additional field research is needed to determine how effective the test entomopathogens would be in controlling *L. occidentalis* populations under field conditions.

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Predbežné výsledky hodnotenia entomopatogénnych húb z radu Hypocreales proti *Leptoglossus occidentalis* (Heteroptera: Coreidae) v laboratórnych podmienkach

Súhrn

Leptoglossus occidentalis, bzdocha pochádzajúca zo Severnej Ameriky, je považovaná za významného škodcu semenných porastov ihličnatých drevín v areáli svojho prirodzeného rozšírenia. V nedávnom období bola táto bzdocha náhodne zavlečená do južnej Európy a jej populácia začala expandovať do ostatných častí kontinentu. Počas prieskumu populácie bzdochy na Slovensku sme identifikovali na mŕtvych jedincoch dva druhy entomopatogénnych húb, *Isaria fumosorosea* a *Beauveria bassiana*. V tejto práci sme v laboratórnych podmienkach hodnotili patogenitu šiestich pôvodných izolátov troch entomopatogénnych húb, *B. bassiana*, *I. fumosorosea* a *Metarhizium anisopliae*, k dospelým jedincom tejto exotickej bzdochy. Všetky testované izoláty preukázali virulenciu voči bzdoche, jej miera však varírovala preukazne medzi jednotlivými izolátmi. Vo všeobecnosti, izoláty získané z prirodzene zabitých bzdôch *L. occidentalis* dosiahli vyššiu patogenitu než tie, čo boli izolované zo vzoriek pôdy. Hodnoty LC_{50} získané probitovou analýzou boli v rozsahu od 0,86 do 84,68 × 10^5 konídií ml^{-1} a izoláty druhu *I. fumosorosea* dosiahli najnižšie hodnoty strednej letálnej koncentrácie (LC_{50}). Výsledky laboratórnych pokusov naznačujú, že huba *I. fumosorosea* má potenciál ako mikrobiálny bioagens pre reguláciu bzdochy *L. occidentalis*.

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