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Natural Prevalence of Entomopathogenic Fungi in Hibernating Pupae of *Cameraria ohridella* (Lepidoptera: Gracillariidae) and Virulence of Selected Isolates

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Abstract

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Spontaneous infection of hibernating *Cameraria ohridella* pupae by entomopathogenic fungi was monitored at two localities of south-western Slovakia and efficacy of selected fungal isolates against the pupae was evaluated in laboratory. Natural prevalence of fungal infection in pupal populations was low (< 7%) and varied depending upon collecting date, locality, year, and pathogen. Ten isolates of three fungal species, *Beauveria pseudobassiana*, *Isaria fumosorosea,* and *I. farinosa*, isolated from the naturally infected pupae and three isolates of non-insect origin of two fungi, *B. bassiana* and *Metarhizium anisopliae*, were screened for colony growth, conidial production *in vitro,* and virulence to *C. ohridella* pupae. A significant variability in the evaluated traits was detected among the isolates. Pupae demonstrated vulnerability to all the isolates irrespective of their origin, however virulence of isolates varied significantly. *I. fumosorosea* was the most virulent fungus with median lethal concentration of 0.09×10^4 conidia/ml (isolate CO10-IFu) and mean survival time of pupae of 7.14 days (isolate CO8-IFu). No correlation between conidial yield of isolates and fungal virulence was observed, but a moderately strong relationship was detected between virulence and mycelial growth rate of isolates.

Keywords: *Beauveria*; horse-chestnut leaf miner; *Isaria*; *Metarhizium*; survival analysis; virulence

The horse-chestnut leaf miner, *Cameraria ohridella* Deschka & Dimić, 1986 (Lepidoptera, *Gracillariidae*), is an invasive species, which causes permanent outbreaks and severely defoliates horse-chestnut (*Aesculus hippocastanum* L.), an important ornamental tree of public parks and gardens in European cities. Since the discovery of *C. ohridella* in Macedonia in 1986 (Deschka & Dimić 1986) its population has gradually invaded most of West and Central Europe. The species has been on the list of 100 worst invasive alien organisms in Europe and its spatial distribution with biology has been thoroughly studied (Kenis *et al.* 2005; Augustin 2009). *C. ohridella* overwinters as a pupa in leaf litter, moths lay eggs on the upper leaf surface from April, and larvae develop in the assimilatory parenchyma of leaves forming typical tunnels (Šefrová & Lastůvka 2001; Kindl *et al.* 2002; Gilbert *et al.* 2005; Augustin *et al.* 2009; Rämert *et al.* 2011; D'Costa *et al.* 2014). A damage caused by larval mining in leaves is spectacular with a serious aesthetic impact on horse-chestnut trees (Gilbert *et al.* 2003; Salleo *et al.* 2003).

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A natural enemy complex of the leaf miner includes parasitoids, predators, and pathogens, but these antagonists are insufficiently adapted and not able to regulate a leaf miner's population density (e.g. Backhaus *et al*. 2002; Grabenweger 2003; Grabenweger *et al*. 2005a, b, 2007; Rämert *et al*. 2011). Control measures of the leaf miner populations focus mostly upon mechanical (Gilbert *et al*. 2003; Kehrli & Bacher 2003; Pavan *et al.* 2003) and chemical control (Kuldová *et al*. 2007), however their effectiveness is either short-term or ecologically questionable. Therefore, an alternative control strategy with the exploitation of entomopathogenic fungi was proposed by Zemek *et al*. (2007).

The hypocrealean entomopathogenic fungi (Ascomycota, Hypocreales) are common parasites of insects or other arthropods helping in prevention of host population outbreaks formation. The fungi can spread fast among host populations horizontally via aerially produced conidia and infect their host by penetrating the cuticle with germ hyphae (Valero-Jiménez *et al.* 2016). Species spectrum and prevalence of these fungi in the leaf miner populations and their effect on the host populations have been only marginally studied (e.g. Samek *et al*. 2006; Prenerová *et al*. 2008; Sierpinska & Kubiak 2011; Metla *et al*. 2013). The fungi can attack all developmental stages of insects, including pupae. Only a few studies on natural fungal parasitism of diapausing pupae during hibernation are available (Samek *et al*. 2006; Prenerová *et al*. 2008; Sierpinska & Kubiak 2011), but general susceptibility of *C. ohridella* pupae to entomopathogenic fungi has been documented by laboratory bioassays (e.g. Richter *et al*. 2007a, b; Kalmus *et al*. 2008). Many strains of entomopathogenic fungi have been isolated and tested against various insect pests and several fungal strains were successfully licensed for commercial use. Some strains of entomopathogenic fungi were tested in laboratory (Richter *et al*. 2007a; Prenerová *et al*. 2009a) or semi-field experiments (Richter *et al*. 2007b; Kalmus *et al*. 2008) against the horse-chestnut leaf miner. One highly virulent strain of *Isaria fumosorosea* Wize, isolated from a *C. ohridella* pupa, was patented (Prenerová *et al*. 2009b, 2013), but it is still far from a practical application.

The goals of this study were (1) to evaluate a natural prevalence of fungal infection in populations of hibernating pupae of *C. ohridella*, (2) to obtain isolates of entomopathogenic fungi from naturally infected individuals, and (3) to test pathogenicity of the fungi to leaf miner pupae in laboratory.

MATERIAL AND METHODS

Prevalence of fungal infection. Incidence of spontaneously infected pupae of *C. ohridella* hibernating in leaf litter was monitored at two localities of south-western Slovakia. The locality of Veľký Cetín (48°13'47''N, 18°10'21''E; 127 m a.s.l.) was visited four times in winter 2013/2014 and twice in autumn 2014. The locality of Dolný Pial (48°07'45''N, 18°27'58''E, 178 m a.s.l.) was visited twice in the late autumn of 2014. At each sampling occasion, a sample of leaf litter (ca. 20 l) was collected. The leaves were inspected and all hibernating pupae were dissected from the leaf tissue. The pupae were incubated in cohorts of 100 individuals in polystyrene Petri dishes (90 × 15 mm) lined with moistened filter paper at 25 ± 1°C in the dark for 14 days. Pupae were monitored daily and those with visible symptoms of fungal infections were separated for microscopic examination and fungus isolation. By microscopic examination, the fungi growing on pupae were identified to a genus level using a key by Humber (2012).

Fungal isolates and conidia preparations. Altogether 26 isolates of entomopathogenic fungi were obtained from infected pupae using a selective culture medium as presented by Medo and Cagáň (2011). The isolated fungi were identified according to the morphology of fungal microstructures (Humber 2012) and during a preliminary screening of the isolates, four *Isaria* and six *Beauveria* isolates were selected based on conidial yield and radial growth rate when cultivated on Sabouraud dextrose agar (SDA; Sigma-Aldrich Chemie GmbH, Darmstadt, Germany). The ten isolates were included in virulence bioassays. Three additional isolates of non-insect origin were also tested in the bioassays. They were two *Beauveria bassiana* (Bals.-Criv.) Vuill. isolates, one endophytic strain (END-BB) isolated from leaf tissue of *Hedera helix* L. and one strain (BOV) isolated from the commercial biopesticide Boverol® (Fytovita s.r.o., Ostrožská Lhota, Czech Republic), and one isolate of *Metarhizium anisopliae* (Metschn.) Sorokin (SO-MA) obtained directly from a soil sample using selective culture medium (Medo & Cagáň 2011). The three isolates were selected in order to compare pathogenicity of isolates from different hosts/sources against *C. ohridella*. The isolate SO-MA demonstrated excellent pathogenicity against lepidopteran larvae in previous laboratory tests (data not published), the isolate BOV has been successfully used against a broad spectrum of pests in agriculture and forestry,

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while pathogenicity of the isolate END-BB to insect hosts has not yet been tested.

The morphological identification of fungal species was supported by rDNA-ITS sequencing study, since the broad overlap in conidial morphology, dimensions and shape among *Beauveria* species complicate definitive identification in this genus. Samples of isolates were disrupted by a bead-beating technique using 2 mm glass beads and DNA was isolated using a classic phenol-chloroform procedure (Sambrook *et al*. 1989). The internal transcribed region (ITS) was amplified using a primer pair ITS5/ITS4 (Medo 2009) and PCR products were sequenced using the ITS5 primer (Macrogen Inc., Seoul, South Korea). Amplified ITS sequences were compared with the GenBank Nucleotide Database (http://www.ncbi.nlm.nih.gov) using the algorithm Blast N (Altschul *et al*. 1997). ITS sequences of the isolates were submitted to GenBank.

The fungal isolates were cultivated on SDA in polystyrene Petri dishes (90 × 15 mm) at 25 ± 1°C in the dark for 10 days. Sporulating cultures were stored at 4°C until use, but not longer than one month. A stock suspension of conidia from each fungal isolate was prepared by suspending a mixture of conidia with mycelium in 200 ml of 0.01% (w/v) aqueous solution of Tween 80® (Sigma-Aldrich Chemie GmbH, Germany). The suspensions were hand-shaken for 1 min and conidia were separated from hyphal debris by filtration through a 10-µm nylon membrane. The concentration of conidia in the suspensions was determined using an improved Neubauer haemocytometer (Brand GmbH, Wertheim, Germany) and required concentrations were obtained by diluting the suspensions in 0.01% Tween 80®. These stock suspensions were used for virulence bioassays with *C. ohridella* pupae and percentage of viable conidia was determined prior to each bioassay. In all cases, more than 95% of conidia were viable as determined by the plate count technique on SDA (Barta 2010).

Conidial yield and radial growth rate tests. A 4-mm-diameter mycelial plug taken from a 10-day-old culture was used for inoculating the centre of SDA plate in a Petri dish (90 × 15 mm). Inoculated dishes were sealed with parafilm and incubated at 25 ± 1°C in the dark. Colony growth was recorded by a digital camera at 24-h intervals during 10 days. Applying methods of the digital image analysis by a freeware software ImageJ Version 1.43u (National Institutes of Health; http://rsbweb.nih.gov/ij/) a size of fungal colony was determined (Barta 2011) and a mean daily colony growth (mm^2/day) was calculated. Three samples including 4-mm-diameter plugs from each plate were taken from the 10-day-old cultures and conidial yield was determined by suspending the conidia in 5 ml 0.05% Tween 80®. The number of conidia was counted using the improved Neubauer haemocytometer (Brand GmbH, Germany). The tests were carried out three times at intervals of two weeks for all isolates.

Insects. Pupae of *C. ohridella* used in the bioassay were dissected from horse-chestnut leaf litter collected at two localities of south-western Slovakia, Veľký Cetín and Vieska nad Žitavou (48°19'05''N, 18°20'52''E; 157 m a.s.l.), during October–December 2014. The pupae were stored in cohorts of 100 individuals in polystyrene Petri dishes (90 × 15 mm) with a moistened cotton plug in cool (4–6°C) and dark conditions until use.

Single-concentration tests. The tests were carried out to evaluate pathogenicity of selected fungal isolates against pupae of *C. ohridella*. For survival analysis, suspensions of 10^6 conidia/ml in 0.01% Tween 80® were prepared. The inoculation of pupae with the fungal conidia was carried out by their dipping into the suspensions. For each isolate, a group of 50 pupae was directly immersed in the conidial suspensions for 5 seconds. An additional group of 50 pupae was immersed in 0.01% Tween 80® as a control. The treated and control pupae were then incubated in transparent polystyrene Petri dishes (90 × 15 mm) lined with wet filter paper for a period of 10 days at 25 ± 1°C and dark conditions. The pupae were monitored at 24-h intervals to record daily mortality. All dead pupae with visible signs of mycosis were incubated separately in Petri dishes (60 mm diameter) with a piece of wet filter paper for 5 days to let the fungi develop and sporulate. Mortality caused by the fungi was confirmed by microscopic examination. The bioassay was repeated three times at intervals of 2–3 weeks for all isolates.

Multiple-concentration tests. For virulence tests, a median lethal concentration of conidia for pupae was estimated from cumulative mortality data at different conidial concentrations. Five isolates were used in the virulence tests, two isolates from the survival analysis displaying a significant effect on viability of pupae and the three isolates of non-insect origin. For each fungal isolate, aqueous conidial suspensions were prepared from the stock suspensions in five concentrations ranging from 10^4 to 10^8 conidia/ml in 0.01% Tween 80®. A group of 100 pupae was treated by dipping in each suspension for 5 seconds. Additional 100 pupae were treated with sterile 0.01% Tween 80® as controls. The treated and control pupae

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were incubated, monitored, and handled as mentioned above. The virulence bioassay was repeated three times under the same conditions at intervals of two weeks.

Data analysis. Cumulative percentage mortality data from a treatment with a standard dose of 10^6 conidia/ml were corrected for a natural (control) mortality using Schneider-Orelli's formula, then arcsine transformed and compared among isolates by the analysis of variance (ANOVA) and Tukey's HSD test if significant differences were detected. ANOVA was also used to determine differences in growth rate and conidia yield among isolates. Possible correlation between radial growth rate, conidia production, and virulence of isolates was analysed through Pearson's correlation coefficient. Mean survival times of pupae treated with isolates at the dose of 10^6 conidia/ml were analysed using survival analysis, where the Kaplan-Meier estimator (Kaplan & Meier 1958) was used to estimate survival functions. All the analyses were performed using Statgraphics Centurion XV (1982–2006 StatPoint Inc.). Cumulative mortality data from the multiple-concentration tests were analysed using Probit analysis (Finney 1971) in Minitab 17 (2013) to estimate lethal concentrations for 50 and 90% mortality (LC_{50} and LC_{90}).

RESULTS

Prevalence of fungal infection. Natural prevalence of entomopathogenic fungi in populations of hibernating pupae was monitored in Veľký Cetín and Dolný Pial during autumn and winter in 2013 and 2014 (Table 1). Altogether 7070 pupae were collected and inspected for a fungal infection. As many as 319 pupae were positive for mycosis and two genera of entomopathogenic fungi, *Beauveria* and *Isaria*, were identified by microscopic examination. The fungi were identified based on morphology of sporulating structures and general symptoms of mycosis. As a whole, the prevalence of fungal disease was low and never exceeded 7% for a particular collecting date and locality. The mean mortality rate of pupae due to fungal infection reached 4.20%; however, the level of mortality varied depending upon collecting date, locality, year, and fungus. Pupae infected with *Beauveria* were more prevalent in populations than those killed by *Isaria*. Out of all mycosed pupae collected, 270 (84.6%) were infected by *Beauveria* spp. and while its prevalence was recorded at similar rate at both localities and in both years, *Isaria* spp. was mostly detected in Veľký Cetín in 2013.

Fungal isolates and their characterisation. Altogether, 26 isolates of entomopathogenic fungi were obtained from infected pupae. Based on a microscopic examination and rDNA-ITS sequencing study of the isolates the following species were identified: *Beauveria pseudobassiana* S.A. Rehner & Humber (16 isolates), *Isaria fumosorosea* (5 isolates), *B. bassiana* (4 isolates), and *Isaria farinosa* (Holmsk.) Fr. (1 isolate). All the cultures were preliminarily screened for conidiation under culture conditions and 10 isolates were selected for colony growth, conidial production, and pathogenicity analyses. A complete list of fungal species and isolates included in the experiments is shown in Table 2.

The radial growth of fungal isolates and the conidial yield varied among and within particular fungal species. The mean radial growth ranged between 5.17 mm^2/day (SO-MA) and 9.83 mm^2/day (CO10-IFu). The mean production of conidia on the surface of

Table 1. Prevalence of fungal infection in the population of *C. ohridella* pupae collected at two localities of south-western Slovakia

Name of locality	Date of collection	No. of pupae collected	Prevalence of infected pupae (%)		
			total	*Beauveria* spp.	*Isaria* spp.
Veľký Cetín	15. 11. 2013	813	2.21	1.23	0.98
	29. 11. 2013	652	5.37	3.22	2.15
	13. 12. 2013	1136	6.87	4.84	2.02
	17. 01. 2014	512	2.15	1.95	0.20
	14. 11. 2014	1305	6.28	6.05	0.23
	28. 11. 2014	1023	3.81	3.81	0
Dolný Pial	02. 12. 2014	721	3.47	3.47	0
	16. 12. 2014	908	3.41	3.19	0.22

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Table 2. Isolates of entomopathogenic fungi included in the bioassays, their production parameters and the closest related species with maximal identities (%) from the BLAST sequence analysis tool

Isolate	Fungal species/GenBank Access. No.	Host (source) of isolate	MCP ± SE	MRGR ± SE	Closest related species with Genbank Access. No.
CO1-BP	*B. pseudobassiana*/KT368173	*C. ohridella* –pupa	9.08 ± 0.83^{c}	9.38 ± 0.58^{a}	*B. pseudobassiana*, NR_111598 (100%)[1]
CO2-BP	*B. pseudobassiana*/KT368170		14.55 ± 0.09^{bc}	7.02 ± 0.31^{ab}	
CO3-BP	*B. pseudobassiana*/KT368172		15.41 ± 0.09^{bc}	6.85 ± 0.48^{b}	
CO4-BP	*B. pseudobassiana*/KT368174		9.52 ± 2.00^{c}	6.26 ± 0.35^{b}	
CO5-BP	*B. pseudobassiana*/KT368171		9.49 ± 0.87^{c}	7.55 ± 0.34^{ab}	
CO6-BP	*B. pseudobassiana*/KT368175		13.37 ± 0.21^{bc}	6.87 ± 0.25^{b}	
CO7-IFa	*I. farinosa*/KT368167		18.41 ± 0.04^{b}	7.92 ± 0.52^{ab}	*I. farinosa*, GU354353 (100%)
CO8-IFu	*I. fumosorosea*/KT368166		27.54 ± 0.16^{a}	9.36 ± 0.48^{a}	*I. fumosorosea*, GU354345 (100%)
CO9-IFu	*I. fumosorosea*/KT368165		31.21 ± 0.20^{a}	8.88 ± 1.03^{a}	
CO10-IFu	*I. fumosorosea*/KT368164		31.87 ± 0.07^{a}	9.83 ± 0.36^{a}	
END-BB	*B. bassiana*/KT368168	*Hedera helix*–leaf	19.32 ± 0.09^{b}	6.99 ± 0.24^{b}	*B. bassiana*, HQ880760 (100%)
SO-MA	*M. anisopliae*/KT368163	soil	27.55 ± 1.59^{a}	5.17 ± 0.38^{c}	*M. anisopliae*, HM055446 (99%)
BOV	*B. bassiana*/KT368169	Boverol®	17.16 ± 1.01^{bc}	7.19 ± 0.29^{ab}	*B. bassiana*, JN379811 (99%)

SE – standard error; MCP – mean conidial production (conidia/mm^2 × 10^5); MRGR – mean radial growth rate (mm^2/day); mean values followed by the same letter within a column are not significantly different at $P = 0.05$ (Tukey's HSD test); [1]maximum identities (%) of sequences from the BLAST sequence analysis tool

SDA varied from 9.08 to 31.87 × 10^5 conidia/mm^2 for CO1-BP and CO10-IFu, respectively (Table 2). Statistical analysis revealed significant differences between the isolates in both the radial growth and the conidial yield (ANOVA; $F_{1.94} = 31.25$, $P < 0.05$ and $F_{1.94} = 35.02$, $P < 0.05$, respectively).

Pathogenicity bioassays demonstrated sensitivity of leaf miner pupae to the selected isolates of entomopathogenic fungi at the dose of 10^6 conidia/ml. Figure 1 shows cumulative mortalities of pupae treated with the fungal isolates. For a majority of isolates, the first pupal mortality occurred on Day 3 and the most rapid increase in cumulative mortality was recorded in Days 5–8 post-treatment. After Day 8, the mortality rate rose only moderately in the test populations and the mean mortality recorded during the Days 9–10 (4.77%) was significantly lower than that for the Days 5–8 (22.35%) or the Days 1–8 (12.01%) of the bioassay ($F_{8.29} = 79.23$, $P < 0.01$ and $F_{8.29} = 31.47$, $P < 0.01$, respectively). The daily mortalities varied significantly ($F_{3.26} = 49.79$, $P < 0.01$) among isolates and on the final day of the bioassay the highest cumulative mortality (79.33%) of pupae was observed for the isolate of *I. fumosorosea* CO10-IFu. The isolate of *B. pseudobassiana* CO2-BP killed the least number of pupae with cumulative mortality of 48.67% on Day 10. In general, *I. fumosorosea* isolates demonstrated the greatest pathogenicity to pupae. These isolates also killed the pupae faster when compared with *Beauveria* isolates. In the case of *I. fumosorosea*, as many as 97–99% of all killed pupae had already been killed before Day 8 post-treatment. For *Beauveria* isolates, the cumulative mortality on Day 8 ranged between 70% and 95%. Control mortality due to entomopathogenic fungi was low reaching 2.67% and only *Beauveria* genus was identified from the control pupae.

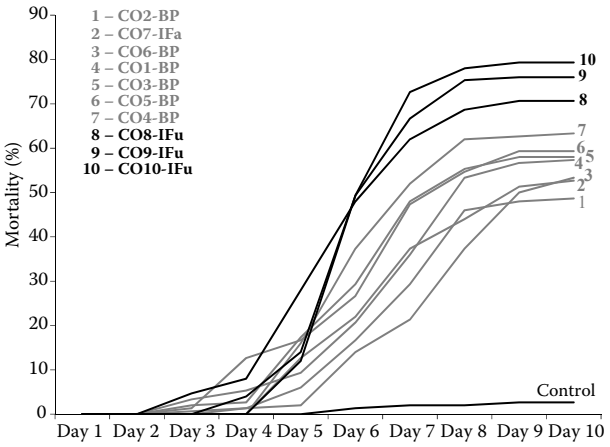

Figure 1. Cumulative mortality (%) of *C. ohridella* pupae treated with suspension of conidia (10^6 conidia/ml) of entomopathogenic fungi

Results of survival analyses testing the effect of fungal isolates on viability of *C. ohridella* pupae over

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Table 3. Results of survival analyses testing the effect of fungal isolates on viability of *C. ohridella* pupae; Kaplan-Meier estimates of cumulative survival probability and mean survival time (MST) of pupae inoculated with conidial suspensions

Isolate	Survival probability (± SE)		MST (± SE)[2]
	Day 5	Day 10	
CO1-BP	0.91 ± 0.03^{bcd}	0.39 ± 0.03^{de}	8.06 ± 0.67^{abc}
CO2-BP	0.94 ± 0.01^{cd}	0.49 ± 0.03^{e}	9.00 ± 0.15^{c}
CO3-BP	0.82 ± 0.02^{b}	0.33 ± 0.02^{bcd}	7.97 ± 0.14^{abc}
CO4-BP	0.84 ± 0.01^{b}	0.24 ± 0.01^{abc}	7.62 ± 0.09^{ab}
CO5-BP	0.85 ± 0.02^{bc}	0.38 ± 0.03^{cde}	8.15 ± 0.11^{abc}
CO6-BP	0.98 ± 0.03^{d}	0.46 ± 0.04^{de}	9.23 ± 0.23^{c}
CO7-IFa	0.87 ± 0.01^{bc}	0.47 ± 0.04^{de}	8.82 ± 0.17^{bc}
CO8-IFu	0.71 ± 0.01^{a}	0.23 ± 0.02^{abc}	7.14 ± 0.05^{a}
CO9-IFu	0.86 ± 0.01^{bc}	0.20 ± 0.03^{ab}	7.25 ± 0.23^{a}
CO10-IFu	0.88 ± 0.01^{bc}	0.18 ± 0.03^{a}	7.20 ± 0.15^{a}

SE – standard error; mean values followed by the same letter within a column are not significantly different at $P = 0.05$ (Tukey's HSD test)

time are shown in Table 3. Log-rank and Wilcoxon tests were run to see if there is any statistical difference among survival curves of the isolates and both the tests confirmed a significant variability ($\chi^2 = 818.15$, $P < 0.05$ and $\chi^2 = 976.33$, $P < 0.05$, respectively). Kaplan-Meier estimates of cumulative survival probability decreased with time as mortality of pupae increased for all isolates. The lowest survival probability (0.18) on Day 10 was estimated for *I. fumosorosea*, the isolate CO10-IFu, and similar probabilities ($F_{3.46} = 8.57$, $P < 0.01$) were also estimated for the further two *I. fumosorosea* isolates or the *B. pseudobassiana* isolate CO4-BP. The highest probability for survival of pupae on the last day of the bioassay (0.49) was estimated for *B. pseudobassiana*, the isolate CO2-BP. Mean survival time of pupae varied between 7.14 and 9.23 days depending upon isolates and the shortest mean survival times were observed for *I. fumosorosea* isolates.

There were no correlations detected between the conidial yield of isolates and either the mean survival time or the cumulative mortality of pupae. On the other hand, a moderately strong relationship was detected between the mycelial growth of isolates and both the mean survival time ($r = -0.633$, $P = 0.0201$) and the cumulative mortality ($r = 0.579$, $P = 0.0381$) of pupae.

The virulence bioassay against pupae of *C. ohridella* was carried out with five fungal isolates, including one isolate of *I. fumosorosea* and *B. pseudobassiana* showing the highest pathogenicity from the survival

Table 4. Results of probit analyses testing virulence of fungal isolates against *C. ohridella* pupae after their exposure to conidial suspensions

	Isolate				
	CO10-IFu	CO4-BP	BOV	END-BB	SO-MA
LC_{50} (× 10^4) ± SE	0.09 ± 0.04^{a}	138.23 ± 16.22^{c}	198.12 ± 24.28^{c}	119.89 ± 17.82^{c}	70.70 ± 13.37^{ab}
95% fiducial CI	0.02–0.30	109.79–174.24	155.53–251.98	89.41–160.58	48.48–102.28
LC_{90} (× 10^6) ± SE	25.94 ± 12.96^{a}	63.50 ± 12.92^{ab}	81.84 ± 17.54^{ab}	179.66 ± 52.91^{ab}	777.49 ± 351.11^{b}
95% fiducial CI	11.04–83.09	43.70–97.45	55.40–129.17	105.56–338.30	349.64–2191.38
Slope ± SE	0.13 ± 0.01	0.33 ± 0.01	0.34 ± 0.02	0.26 ± 0.01	0.18 ± 0.01
Constant ± SE	–0.87 ± 0.17	–4.73 ± 0.20	–4.99 ± 0.25	–3.58 ± 0.19	–2.46 ± 0.16
P-value	0.000	0.000	0.000	0.000	0.000
Conidial viability ± SE (%)	95.50 ± 1.19	95.04 ± 2.25	96.03 ± 0.98	96.50 ± 1.91	96.20 ± 0.81

SE – standard error; values of lethal concentrations and 95% fiducial confidence intervals (CI) are in conidia/ml suspension; values followed by the same letter in the row are not significantly different at $P = 0.05$ (Tukey's HSD test)

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analysis (CO10-IFu and CO4-BP, respectively), as well as the three isolates obtained from substrates other than *C. ohridella*. The basic measure of virulence generated in the virulence bioassay was lethal concentration of conidia LC_{50} and LC_{90} expressed as a number of conidia/ml in the test suspensions inducing 50 and 90% mortality of treated pupae on day 10 post-inoculation, respectively. The percentage mortality of pupae increased with the conidial concentration in suspensions that allowed estimating a dose-response relationship by the probit analysis. The lethal concentration values (LC_{50} and LC_{90}) of the five isolates are presented in Table 4. The results clearly demonstrate an inter-specific variability in virulence, when significant differences were detected among the isolates (ANOVA; $F_{5.99} = 9.66$, $P < 0.01$ for LC_{50} and $F_{3.48} = 7.23$, $P < 0.05$ for LC_{90}). The lethal concentration values, as estimated by the probit analysis, ranged from 0.09 to 198.12×10^4 conidia/ml for LC_{50} and from 25.94 to 777.49×10^6 conidia/ml for LC_{90}. According to the lethal concentration values, *I. fumosorosea* isolate CO10-IFu was the most effective on pupae and *B. bassiana* isolate BOV (LC_{50}) or *M. anisopliae* isolate SO-MA (LC_{90}) were the least virulent.

DISCUSSION

Entomopathogenic fungi from the order Hypocreales are common and omnipresent soil-born insect pathogens, which can kill a variety of insect hosts (Vega *et al*. 2012). Overwintering stages of many insects, including leaf miners, are exposed to soil or plant debris, the natural deposits of fungal inocula, for a variously long period. Therefore, mycoses in populations of such insect stages belong among common mortality factors in natural habitats and infection rates may vary in host populations. Natural mortality of leaf miner pupae due to fungal infection detected during our observations confirmed an activity of these insect pathogens at the localities of south-western Slovakia. The mean mortality rate of 4% is low, but corresponds to results of similar studies. In Latvia, mortality rate of *C. ohridella* larvae was even lower reaching 0.9–1.6%. However, a species spectrum of fungi was broader, when fungi belonging to six genera were identified, including *Aspergillus*, *Hirsutella*, *Beauveria*, *Metarhizium*, *Lecanicillium*, and *Isaria* (Metla *et al*. 2013). In Poland (Warsaw), three genera of fungi, *Beauveria*, *Isaria*, and *Lecanicillium*, were identified and isolated from *C. ohridella* pupae (Sierpinska & Kubiak 2011), but *Lecanicillium* was the only entomopathogenic fungus determined from overwintering pupae of *C. ohridella* in the Czech Republic (Samek *et al*. 2006). We identified four fungal species out of two genera from infected pupae. The detection of *B. pseudobassiana* as the predominant *Beauveria* species isolated from infected pupae is an interesting fact, since this fungus is usually considered the less common *Beauveria* species, especially in arable sites. For example, Pérez-González *et al*. (2014) found only four *B. pseudobassiana* isolates out of 40 *Beauveria* isolates from agricultural soil in Mexico. A different species structure of *Beauveria* was presented by Medo (2009) from soil samples in Slovakia. Out of 109 *Beauveria* isolates obtained, 56 isolates were identified as *B. bassiana* and 47 isolates were *B. pseudobassiana*. Moreover, a habitat preference of different *Beauveria* genotypes isolated from soil in Slovakia was observed. While *B. pseudobassiana* predominated in forest habitats, *B. bassiana* was more common in arable soil. The author, however, was not able to confirm unequivocally that *B. pseudobassiana* is a species with rigorous inclination to natural habitats like forests, including forest edges and hedgerows (Medo 2009). On the other hand, habitat preference of entomopathogenic fungi is well known and supported by several studies (Vanninen 1996; Bidochka *et al*. 1998; Quesada-Moraga *et al*. 2007). Generally, a more common and frequently identified *Beauveria* species from insect hosts is *B. bassiana*. Since *B. pseudobassiana* is a new taxon, morphologically very similar to *B. bassiana*, and was a part of *B. bassiana* species complex before 2011 (Rehner *et al*. 2011), its prevalence in insect population might be underestimated. In previous works it was probably identified as *B. bassiana*. Basic studies on ecology and distribution of recently described *B. pseudobassiana* are limited, therefore we are not able, at present, to define which factors, if any, might favour the preference of this fungus to *C. ohridella* pupae. Since we determined to a species level only a small part of *Beauveria*-killed pupae, we cannot exclude a higher activity of *B. bassiana* within the populations of *C. ohridella* pupae.

Our laboratory bioassays demonstrate that all isolates of the tested fungi were pathogenic and induced infection to hibernating pupae of *C. ohridella*. An optimum incubation period of 10 days post-treatment was selected as necessary to assess the efficacy of fungi. Selection of a suitable incubation time is criti-

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cal to a bioassay procedure taking into consideration the infection cycle of the tested fungus. Many factors enter the host–pathogen interaction and influence the progress of infection cycle (Valero-Jiménez *et al.* 2016). Besides environmental factors, like temperature and humidity, also the host's fitness and size, as well as the number of infection particles (a dose of conidia) that get in contact with host's cuticle should be taken into consideration. In many virulence tests > 12 days of incubation period are used to ensure that all mortality resulting from infection is expressed. However, because there was no significant increase in cumulative mortality after Day 8 of our survival analyses, we could shorten the bioassay period to 10 days. The results of survival analyses demonstrate that the most rapid increase in cumulative mortality occurred between Days 5 and 8 after treatment and within the first 8 days a majority of pupae died. As expected, there was a considerable variation in virulence among fungal isolates. Such variations in virulence among strains of entomopathogenic fungi are well known and have been documented for *B. bassiana* (Jones *et al*. 1996; Talaei-Hassanloui *et al*. 2006; Medo 2009), *I. fumosorosea* (Shapiro-Ilan *et al*. 2008), or *M. anisopliae* (Jones *et al*. 1996). Generally, it is considered that the most virulent isolates of entomopathogenic fungi to an insect host are those that have been isolated from the same or related host species (Goettel *et al*. 1990). This was also observed in the present bioassays, when the isolates of non-leaf miner origin (BOV, END-BB, and SO-MA) demonstrated a lower virulence. Faster growing isolates are considered to have an advantage as biocontrol agents because this allows less time needed for inoculum production, provides a competitive domination over other microorganisms during a saprophytic stage of pathogenesis, and shortens infection cycle in hosts which can make the pest control more effective (Varela & Morales 1996). In our laboratory study, mycelial growth rate of tested isolates correlates with mean survival time and cumulative mortality of pupae. However, this kind of correlation was not detected for *B. bassiana* in other studies (Talaei-Hassanloui *et al*. 2006).

As presented in laboratory bioassays of this study, the entomopathogenic fungi possess a great potential for biocontrol of leaf miner pupae. The Slovak isolate of *I. fumosorosea* CO10-IFu demonstrated promising pathogenic attributes against pupae of horse-chestnut leaf miner in controlled laboratory conditions, but further semi-field and field testing is needed before it can be recommended for a practical use. In addition, its suitability for mass-production, formulation and virulence to other life stages of the pest and its parasitoids have to be evaluated.

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