Article

Hypocrealean Fungi Associated with *Hylobius abietis* in Slovakia, Their Virulence against Weevil Adults and Effect on Feeding Damage in Laboratory

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Abstract: In temperate regions of Europe, the large pine weevil, *Hylobius abietis*, is a major pest of coniferous forests mostly at sites where clear-felling is followed by planting of saplings. Control measures against this pest are based on silvicultural techniques, an application of physical barriers on stems of saplings and insecticide treatments. To avoid the use of insecticides, alternative measures such as biological control have been investigated. The goal of the present study was to obtain local strains of entomopathogenic fungi (Ascomycota, Hypocreales) from natural populations of *H. abietis*, and to investigate their efficacy against the weevil. A survey on entomopathogenic fungi was undertaken at clear-felled areas of spruce forests in northern Slovakia. Two *Beauveria* species, *B. bassiana* and *B. pseudobassiana*, were identified, and 22 in vitro strains were obtained. Mean prevalence of infected adults was low ($2.10\% \pm 0.67\%$) and the mycosis was mostly recorded during May and June. Virulence of *Beauveria* strains against the weevil was tested in laboratory. *B. bassiana* strain AMEP20 was significantly most virulent (LC_{50} of $0.65 \pm 0.10 \times 10^8$ conidia mL^{-1}). Treatment with conidia of AMEP20 strain affected feeding damage by the weevil on bark of Scots pine twigs. Daily bark consumption by *B. bassiana*-treated weevils was lower than by untreated individuals and decreased with increasing conidia concentration used for the treatment. In the outdoor experiment, AMEP20 strain killed weevils that fed on spruce saplings treated with conidia suspensions. Mortality due to mycosis on weevils exposed to the conidia-treated saplings reached 30.0%–76.5% and 55.0%–88.2% after 32 and 46 days, respectively.

Keywords: biocontrol; entomopathogenic fungi; *Hylobius abietis*; Hypocreales; virulence

1. Introduction

The large pine weevil, *Hylobius abietis* L. (Curculionidae: Molitrynae), is regarded as one of the most damaging pests of newly planted or naturally regenerating coniferous forests across Northern and Western Europe [1,2]. This Palearctic species is naturally distributed from the north-west of Europe to Siberia [3]. Larvae develop inside cambial tissues of underground parts of stumps of felled coniferous trees, and adults feed on bark of young saplings. Adult weevils may cause considerable economic loss by damaging the bark, what often leads to sapling mortality, or reduced growth with stem deformations. In some cases, a complete destruction of new plantations occurs, especially where clear-felling is immediately followed by planting of coniferous saplings [1,2,4–6]. A single weevil can damage several saplings [7]. Thus, a relatively low number of adults can have a significant impact on regeneration of

reforestation areas. Moreover, weevils can migrate more than 10 km and can therefore cause damage over a broad area [8]. In laboratory assays, a polyphagous habit of adults was demonstrated, although coniferous species were always preferred [9]. While a range of coniferous host tree species is wide [10], Scots pine (*Pinus sylvestris* (L.)) and Norway spruce (*Picea abies* (L.) H. Karst) are the main hosts [11].

Thousands of hectares of reforestation sites are annually threatened by *H. abietis* in Europe [12]. If the sites are left unprotected, economic losses to the forest industry can be severe. In Slovakia, *H. abietis* population density has been considerably increasing since 2010 and spruce saplings in reforestation areas have been significantly damaged [13–15]. Control measures against *H. abietis* are mostly based on a range of silvicultural techniques (e.g., removal of breeding material after felling, delayed planting, shelter-wood planting system, mixed planting, and timing of felling), and an application of physical barriers on stems of saplings before or at planting (e.g., latex paint, PVC (polyvinyl chloride) barriers, sand (Conniflex) and glue based stem coating, and wax coating) [2,16–25]. The curative insecticide application is usually not efficient due to a sheltered lifecycle of larval stage [26]. Therefore, the preventive use of insecticides on saplings before planting is the main control measure currently employed in most countries, which practice reforestation [2,27]. Because weevils are able to detect some insecticides (pyrethroids and nicotinoids) and to avoid treated saplings, insecticide treatments protect young saplings, but have little impact on local populations of the pest [18,26].

Although the pesticide use in forestry is small compared to agriculture and horticulture (on average 0.10%–1.10% of total volume used in Slovakia during 2000–2015 [28]), its minimization and reducing the risk of damage to the environment is an important task of an integrated approach for managing forest pests. To avoid the use of insecticides, alternative measures such as biological control and their suitability for the *H. abietis* control have been investigated. Several natural antagonists of pine weevils, including entomopathogenic nematodes, fungi and microsporidia, have been studied as potential biocontrol agents [29–35]. Over the last two decades, more attention was put to nematodes (families Steinernematidae and Heterorhabditidae) for inundative biocontrol targeted against pine weevil larvae, pupae, and callow adults [29,30,32,34]. Although nematodes can reduce numbers of emerging *H. abietis* adults, there is an increasing interest to apply entomopathogenic fungi (Ascomycota, Hypocreales) against larvae and pupae, since they possess several properties, which make them more suitable for weevil control. They infect all developmental stages, have a better shelf-life of inoculum, have a longer persistence in the field with a potential to be augmented by mycosed individuals, and finally a fungal inoculum application is technically less complicated than in the case of nematodes [36].

Entomopathogenic fungi (EPF) are natural antagonists of arthropods helping in control of host population and prevention of outbreaks formation [37]. There has been an extensive research on the use of fungal entomopathogens as perspective biocontrol agents of insect pests in agriculture and forestry [38], and some strains have been successfully licensed and commercialised [39,40]. These fungi are considered to be environmentally safe [41], can be mass-produced [42], and show a considerable potential to control various forest insect pests [43]. In Europe, several reports have focused on the occurrence of EPF in *H. abietis* populations, and species of *Beauveria* and *Metarhizium* genera have been reported from larvae and adults. Generally, the fungi are associated with *H. abietis* populations at a relatively low, though a constant prevalence level [32,35,44,45]. Results of earlier and recent attempts to use EPF against *H. abietis* in laboratory were either inconsistent or not very promising [32,36,46–50]. In spite of that, the fungi are believed to have a potential to be successfully implemented into an integrated system managing the problem of pine weevil damage. Feasibility and sustainability of their use as control agents of *H. abietis* in the field depend on the choice of fungal strains, inoculum formulations, and application techniques.

The present study aims to identify entomopathogenic fungi in natural populations of *H. abietis* in Slovakia, to obtain local strains of the fungi and to investigate their virulence against the weevil in laboratory and semi-field bioassays with testing their effect on feeding damage.

2. Materials and Methods

2.1. Collecting and Handling of Weevils

Pitfall traps [4,5,51,52] baited with 96% ethanol and 50 mm long pieces of Scots pine twigs were used for trapping *H. abietis* adults at several clear-felled areas of spruce forests in northern Slovakia. Most of trappings were conducted at the High Tatra Mountains in the Tatra National Park (49°10'0" N, 20°08'0" E) and at the Low Tatra Mountains (48°57'0" N, 19°30'0" E) during 2015–2018, where generally high damage by the weevils had been determined during our earlier investigations. During 2015–2017 the trapping was neither extensive nor systematic and traps were installed and checked irregularly. In 2018, the traps in the Tatra National Park were checked regularly once a week from the end of April to September to determine the prevalence of fungal infection in pest population. All trapped weevils were placed into rectangular plastic boxes (250 × 150 × 150 mm) in groups of 50 individuals per box and incubated (20 °C and 80% relative humidity (RH)) for 21 days to observe a natural prevalence of entomopathogenic fungi. Each dead weevil displaying typical macroscopic symptoms of fungal infection was placed in a 1.5 mL microtube and stored at 5 °C until used for obtaining fungal cultures. Dead adults displaying no external symptoms were incubated individually in Petri dishes (60 × 15 mm) on a piece of wet filter paper at 25 °C for 72 h to stimulate fungal growth. All cadavers with characteristic symptoms of mycosis were examined under a dissecting microscope (40×) to detect possible contaminants or death caused by other factors. Fungal cultures were obtained from the individuals with confirmed fungal infection.

For virulence bioassays, collected weevils were separated according to sex and kept in plastic boxes (150 × 10 × 80 mm) at 5–7 °C for a maximum of two months prior to their use. The weevils were provided with fresh food (Scots pine twigs) and kept at 20 °C and 60 ± 10% RH 48 h before the experiments.

For bioassays with a measurement of feeding damage, the weevils separated to males and females starved at 20 °C and 60 ± 10% RH for 48 h before the experiment to ensure the consistency in feeding damage between replicates.

2.2. In Vitro Isolation and Identification of Entomopathogenic Fungi

In vitro isolates of EPF were obtained from infected weevils using a selective culture medium (Sabouraud-dextrose agar (SDA) supplemented with 600 mg·L⁻¹ streptomycin sulphate, 50 mg·L⁻¹ tetracycline hydrochloride, 250 mg·L⁻¹ cyclohexamide, and 500 mg·L⁻¹ dodine (all chemicals from Sigma-Aldrich®, Saint Louis, MI, USA)). Isolated colonies were sub-cultured on SDA without antibiotics and fungicides in culture glass tubes at 25 ± 1 °C in the dark for 10 days and sporulating cultures were stored at 5 °C. All obtained isolates were deposited in the fungal collection of the Institute of Forest Ecology of the Slovak Academy of Sciences (Nitra, Slovakia).

The in vitro cultures were microscopically (500×) identified to a genus level according to morphology of microstructures [53–55]. The morphological identification was supplemented by a sequencing study of internal transcribed region (ITS) of rDNA and a partial sequence of TEF1- α gene. DNA of fungi was isolated using EZ-10 Spin Column Fungal Genomic DNA Kit (Bio Basic Canada Inc., Ontario, Canada) according to the manufacturer's instructions. The ITS region was amplified with a primer pair ITS1-F/ITS4 [56,57] and the TEF-1 α gene with primers 983F and 2218R [54]. PCR products were purified by QIAquick PCR Purification Kit (Qiagen n.v., Venlo, Netherlands) and sequenced using the primers ITS4 or 983F (Macrogen Europe Inc., Amsterdam, The Netherlands). Obtained sequences were subjected to Blast N [58] against GenBank Nucleotide Database for taxonomic classification. All sequences obtained from this study were deposited in GenBank (Table 1).

2.3. Fungal Strains

Fungal strains obtained from naturally killed *H. abietis* (Table 1) were cultivated on Sabouraud-dextrose agar (SDA) in polystyrene Petri dishes (94 × 16 mm) at 25 ± 1 °C in the dark for 5 days and

under the continuous light for the next 5 days. The 10-day-old sporulating cultures were stored at 4 °C prior to their use in bioassays, but not longer than two weeks. A percentage of viable conidia was determined prior to each bioassay by germination tests on agar plates [59]. Conidial viability of all isolates used for tests was >95% ($\bar{x} = 96.63 \pm 0.20\%$) measured after 12 h incubation on an agar plate at 25 °C.

Table 1. List of *Beauveria* strains isolated from naturally infected adults of *Hylobius abietis* in Slovakia during 2015–2018.

Strain	Fungal Species	GenBank Accession No. ¹	
		ITS	TEF-1 α
AMEP020	<i>Beauveria bassiana</i>	MK490859	MK504338
AMEP043	<i>Beauveria pseudobassiana</i>	MK490860	MK504354
AMEP052	<i>Beauveria bassiana</i>	MK490861	MK504339
AMEP053	<i>Beauveria bassiana</i>	MK490862	MK504340
AMEP056	<i>Beauveria bassiana</i>	MK490863	MK504341
AMEP067	<i>Beauveria pseudobassiana</i>	MK490864	MK504355
AMEP072	<i>Beauveria bassiana</i>	MK490865	MK504342
NREP083	<i>Beauveria pseudobassiana</i>	MK490866	MK504356
NREP084	<i>Beauveria pseudobassiana</i>	MK490867	MK504357
NREP087	<i>Beauveria bassiana</i>	MK490868	MK504343
NREP088	<i>Beauveria bassiana</i>	MK490869	MK504344
NREP090	<i>Beauveria bassiana</i>	MK490870	MK504345
NREP091	<i>Beauveria bassiana</i>	MK490871	MK504346
NREP094	<i>Beauveria bassiana</i>	MK490872	MK504347
NREP095	<i>Beauveria bassiana</i>	MK490873	MK504348
NREP096	<i>Beauveria bassiana</i>	MK490874	MK504349
NREP097	<i>Beauveria bassiana</i>	MK490875	MK504350
NREP098	<i>Beauveria bassiana</i>	MK490876	MK504351
NREP099	<i>Beauveria pseudobassiana</i>	MK490877	MK504358
NREP100	<i>Beauveria bassiana</i>	MK490878	MK504352
NREP102	<i>Beauveria bassiana</i>	MK490879	MK504353
NREP103	<i>Beauveria pseudobassiana</i>	MK490880	MK504359

¹ GenBank Accession numbers of DNA sequences for ITS and TEF-1 α regions submitted to GenBank database.

2.4. Virulence Bioassays against *H. abietis* Adults

A stock suspension of conidia was prepared by suspending a mycelial mat in 250 mL of 0.01% (w/v) Tween®80 inside a 500 mL reagent flask. The flask was hand-shaken for 60 s and mycelial debris were removed from the suspension by filtration through a sterile 10 μ m nylon membrane (Spectra Mesh®, Spectrum Chemical Mfg. Corp., New Brunswick, NJ, USA). Concentration of conidia was determined using an improved Neubauer hemocytometer, and the required concentration of the stock suspension was obtained by dilution in 0.01% (w/v) Tween®80. The conidia suspensions were used immediately for virulence bioassays. A median lethal concentration of conidia (LC₅₀) for *H. abietis* was estimated from cumulative mortality data at four different conidia concentrations ranging from 1×10^6 to 1×10^9 conidia mL⁻¹ in 0.01% (w/v) Tween®80. Groups of 15 males and females were treated by their submersion in the suspensions for 10 s, and additional 15 males and females were treated with sterile 0.01% Tween®80 as controls. Each group of treated and control weevils was incubated in Petri dishes (200 $\times$ 35 mm) lined with wet filter paper at 22 ± 1 °C, saturated humidity and 12/12 h (L/D) photoperiod for 21 days. Five sections of Scots pine twigs (100–150 mm) were provided as food to each Petri dish. The pine twigs were superficially disinfected by germicidal (UV-C) irradiation for 30 min under aseptic conditions of a laminar flow cabinet before their use as food. The weevils were monitored at 24 h intervals to record their daily mortality. All cadavers were incubated separately in sterile Petri dishes (60 $\times$ 15 mm) on a piece of wet filter paper for 5 days to facilitate fungal development. Mortality caused by the fungi was confirmed by microscopic examination and only cadavers with confirmed

infection were used to estimate virulence. Three most virulent *Beauveria* strains were selected and used to repeat the bioassay under the same conditions five times.

2.5. Feeding Damage Bioassay

The most virulent *B. bassiana* strain (AMEP20) was used in a laboratory bioassay to measure an effect of the fungal treatment on feeding damage by the weevils. Adults (15 males and 15 females) were individually inoculated by conidia suspensions at four concentration levels (from 1×10^5 to 1×10^8 conidia mL^{-1}) as described above, and additional groups of 15 males and 15 females were treated with sterile 0.01% (w/v) Tween®80 as controls. The inoculated and control weevils were incubated individually in Petri dishes (200 × 35 mm) with wet filter paper on the bottom at 22 ± 2 °C and natural photoperiod. A freshly cut twig of Scots pine (approximately 100-mm-long and 7-mm-thick) was put into each Petri dish. The weevils were monitored for 46 days. Mortality was recorded and pine twigs were replaced by fresh ones on the day 11, 22 and 33. The amount of feeding damage on the twigs was assessed with a modified method by Leather et al. [60]. A damage outline on the twigs was traced onto a piece of transparent graph paper twisted around the surface of twigs with a black pen. The number of millimetre squares occluded by the trace pattern was counted, and the damaged area was calculated for each twig. Dead weevils were processed as described in the virulence bioassay. The experiment was carried out in three repetitions.

2.6. Outdoor Bioassay with *B. bassiana* Treatment

To simulate field conditions, an outdoor trial was set-up to evaluate the effect of *B. bassiana*-treatment on *H. abietis* adults. Two-year-old Norway spruce (*Picea abies*) saplings (25–30 cm high) were planted in pots (10 cm diam., 470 mL) and equally spaced in outdoor conditions. A group of 147 saplings was treated with conidia suspension (1×10^8 conidia mL^{-1}) of AMEP20 strain using a hand atomizer. Each sapling was treated with 0.95 mL of the suspension. A group of 21 saplings was treated in the same way with sterile 0.01% (w/v) Tween®80 as controls. Another group of 21 saplings was also treated with sterile 0.01% (w/v) Tween®80 as mentioned above. This group was used for a direct treatment trial. Every treated plant was inserted into a sleeve made of white polyamide mesh (aperture of 0.5 mm) to prevent *H. abietis* adults from escaping. A single unsexed adult of *H. abietis* was released to each of 21 control saplings and 21 suspension-treated saplings on the day of treatment (day 0). During the following six days (days 1–6), further groups of 21 suspension-treated saplings were infested with *H. abietis* adults every day. Together, 147 suspension-treated and 21 control saplings were infested with a single *H. abietis*. The group of 21 Tween®80-treated saplings were infested on the day 0 with a single *H. abietis*, which had been inoculated with conidia suspensions (1×10^8 conidia mL^{-1}) as described in the virulence bioassay (a direct treatment). Mortality of *H. abietis* was checked twice during the bioassay, 32 and 46 days after the saplings were infested with weevils. The bioassay started on 12 May and ended on 27 June 2018. Mean daily temperature varied from 11.9 to 21.0 °C ($\bar{x} = 16.7$ °C) during the bioassay, and daily precipitation total ranged from 0 to 8.8 mm ($\Sigma = 30.4$ mm). Daily temperature and precipitation data that were obtained from a local automatic meteorological station are displayed in Figure 1.

2.7. Data Analysis

Cumulative mortality data from the virulence tests were corrected for natural (control) mortality using Schneider–Orelli's formula and subjected to probit analysis to estimate median lethal concentrations (LC_{50}) with associated 95% confidence intervals. A LC_{50} ratio [61] was calculated for each fungal strain to determine whether LC for females significantly differed from LC for males. Lethal concentrations for the most virulent strains were subjected to ANOVA and the post-hoc Tukey's HSD test was performed to separate and compare means if significant differences ($\alpha = 0.05$) were detected. Chi-square test was used for testing a hypothesis that there is no difference ($\alpha = 0.05$) in the mortality occurrence between *H. abietis* adults directly treated with conidia suspension and adults exposed to

saplings treated with conidia in the outdoor bioassay. All the analyses were conducted using Minitab 17® (© 2013 Minitab Inc., State College, PA, USA).

Figure 1. Daily mean temperature (°C) and daily precipitation (mm) data measured during the outdoor experiment.

3. Results

3.1. Occurrence of Entomopathogenic Fungi in Populations of *H. abietis*

During the four-year investigation on the occurrence of EPF in populations of *H. abietis*, weevils killed by *Beauveria* sp. were observed. As many as 53 cadavers were inspected and *Beauveria* sp. was identified in 42 of them. Altogether, 22 in vitro isolates of EPF (Table 1) were obtained from the inspected cadavers and two *Beauveria* species were identified, namely *B. bassiana* (Bals.-Criv.) Vuill. (16 isolates) and *B. pseudobassiana* S.A. Rehner and R.A. Humber (6 isolates). Prevalence of mycosis in host populations was only quantified at the locality of the Tatra National Park in 2018. During this year, out of 1188 trapped adults, 25 individuals (2.10%) displayed symptoms of *Beauveria* infection that was confirmed by microscopic and molecular techniques. Incidence of infected adults was low in population and varied between 0 and 5.97% depending on collecting date. Infected individuals were mostly found in May and June and prevalence of disease culminated in the second half of June. No mycosis was observed after July 7.

3.2. Virulence of Fungal Strains against *H. abietis* Adults

Pathogenicity of the *Beauveria* strains against weevils was tested in a series of laboratory bioassays. Percentage cumulative mortality increased with conidia concentrations and varied among fungal strains. Depending on strains, cumulative mortality reached 0–26.67% ($\bar{x} = 9.55\% \pm 0.90\%$) at the lowest conidia concentration (1×10^6 conidia mL⁻¹) and 46.67%–86.67% ($\bar{x} = 63.94\% \pm 1.52\%$) at the highest concentration (1×10^9 conidia mL⁻¹). Mean cumulative mortality in the control was $1.21\% \pm 0.08\%$ for males and $0.53\% \pm 0.05\%$ for females. Median lethal mortality (LC₅₀) varied among the strains (Table 2) and ranged from 0.52 to 13.28×10^8 conidia mL⁻¹. Low variability in LC₅₀ values between males and females was detected but LC₅₀ ratio analysis showed no significant differences (Table 2). The most virulent strains of *B. bassiana* (AMEP20) and *B. pseudobassiana* (AMEP43 and NREP84) with estimated LC₅₀ around 1×10^8 conidia mL⁻¹ were selected to repeat the virulence bioassay five times. In this case, since sex of weevils had no effect on susceptibility to fungal infection ($F_{(1,8)} = 0.34$, $p > 0.05$ for AMEP20; $F_{(1,8)} = 0.74$, $p > 0.05$ for AMEP43; $F_{(1,8)} = 0.25$, $p > 0.05$ for NREP84), mortality data for males and females were pooled before subjecting to probit analysis. Mean LC₅₀ values for the three most virulent strains are shown in Table 3. AMEP20 strain with LC₅₀ of $0.65 \pm 0.10 \times 10^8$ conidia mL⁻¹ was significantly ($F_{(2,12)} = 31.01$, $p < 0.01$) more virulent than the other two strains.

Table 2. Summary of probit analysis parameters from the virulence bioassays performed with *Beauveria* spp. strains against males (M) and females (F) of *Hylobius abietis* in laboratory.

Strain	Sex	LC ₅₀ ± SE ¹ × 10 ⁸	95% Fiducial Confidence Interval ¹	Slope ± SE ²	LC ₅₀ Ratio ³	95% Confidence Interval ³
AMEP20	M	0.52 ± 0.03	0.07–1.71	0.36 ± 0.10	38.42	0.24–610
	F	0.78 ± 0.06	0.08–4.30	0.29 ± 0.09		
AMEP43	M	1.05 ± 0.12	0.22–3.17	0.42 ± 0.11	2.52	0.10–62.46
	F	1.07 ± 0.10	0.21–3.51	0.40 ± 0.11		
AMEP52	M	1.88 ± 0.11	0.04–8.56	0.39 ± 0.12	0.88	0.02–47.75
	F	2.23 ± 0.14	0.06–11.56	0.36 ± 0.10		
AMEP53	M	6.89 ± 0.40	0.18–107.91	0.56 ± 0.23	1.60	0.01–307
	F	5.51 ± 0.40	0.16–71.72	0.40 ± 0.12		
AMEP56	M	8.07 ± 0.89	0.13–904.89	0.31 ± 0.13	98.44	0.08–1059
	F	9.79 ± 0.13	0.14–736.54	0.25 ± 0.11		
AMEP67	M	4.21 ± 0.35	0.08–136.59	0.34 ± 0.13	2.17	0.01–1235
	F	4.29 ± 0.37	0.09–130.50	0.30 ± 0.10		
AMEP72	M	10.28 ± 0.70	0.27–316.51	0.58 ± 0.29	1.03	0.01–812
	F	8.12 ± 0.73	0.19–470.10	0.36 ± 0.12		
NREP83	M	3.15 ± 0.19	0.08–16.75	0.43 ± 0.14	14.10	0.01–425
	F	1.93 ± 0.13	0.05–11.16	0.34 ± 0.10		
NREP84	M	1.01 ± 0.19	0.09–8.46	0.27 ± 0.10	1.24	0.02–88.43
	F	0.76 ± 0.05	0.11–3.10	0.33 ± 0.10		
NREP87	M	4.97 ± 0.28	0.13–35.86	0.52 ± 0.20	4.16	0.03–676
	F	4.36 ± 0.34	0.11–69.33	0.34 ± 0.11		
NREP88	M	11.73 ± 0.14	0.20–306.56	0.34 ± 0.16	0.49	0.01–162
	F	13.28 ± 0.16	0.25–226.92	0.32 ± 0.12		
NREP90	M	10.28 ± 0.70	0.27–313.53	0.58 ± 0.29	1.63	0.02–129
	F	10.34 ± 0.88	0.27–451.33	0.41 ± 0.14		
NREP91	M	4.03 ± 0.22	0.11–18.29	0.51 ± 0.17	1.09	0.07–16.33
	F	2.04 ± 0.11	0.06–7.90	0.41 ± 0.11		
NREP94	M	2.66 ± 0.16	0.58–12.42	0.44 ± 0.15	1.43	0.06–36.95
	F	2.23 ± 0.14	0.06–11.56	0.36 ± 0.10		
NREP95	M	8.32 ± 0.50	0.26–83.23	0.59 ± 0.21	1.05	0.10–11.27
	F	6.45 ± 0.38	0.22–41.89	0.50 ± 0.15		
NREP96	M	2.17 ± 0.16	0.04–18.66	0.33 ± 0.11	0.62	0.01–35.02
	F	1.96 ± 0.13	0.05–11.76	0.33 ± 0.10		
NREP97	M	5.09 ± 0.43	0.10–303.90	0.35 ± 0.14	3.45	0.18–65.89
	F	6.53 ± 0.63	0.14–584.54	0.31 ± 0.11		
NREP98	M	3.00 ± 0.17	0.07–132.97	0.47 ± 0.15	38.98	0.01–208
	F	2.63 ± 0.18	0.07–190.81	0.36 ± 0.11		
NREP99	M	9.88 ± 0.59	0.29–117.43	0.65 ± 0.29	68.92	0.05–886
	F	5.75 ± 0.37	0.18–481.19	0.45 ± 0.14		
NREP100	M	3.14 ± 0.18	0.07–157.04	0.47 ± 0.16	1.69	0.01–523
	F	1.63 ± 0.11	0.04–8.54	0.33 ± 0.10		
NREP102	M	5.09 ± 0.43	0.10–303.92	0.35 ± 0.14	0.14	0.01–12.64
	F	3.33 ± 0.22	0.09–247.68	0.37 ± 0.11		
NREP103	M	2.97 ± 0.17	0.08–124.13	0.46 ± 0.14	4.14	0.06–279
	F	2.23 ± 0.14	0.06–115.63	0.36 ± 0.10		

¹ Median lethal concentrations (LC₅₀) and 95% fiducial confidence intervals from probit analysis are in conidia per milliliter of suspension; SE—Standard error of the mean; ² Slope from the regression analysis with standard error (SE); ³ LC₅₀ ratios between males and females of *H. abietis* with 95% confidence intervals [61]; Mean control mortality in the bioassays reached 1.21% ± 0.08% for males and 0.53% ± 0.05% for females.

Table 3. Summary of probit analysis parameters from the virulence bioassays performed with the most pathogenic *Beauveria* strains against *Hylobius abietis* adults in laboratory.

Strain	LC ₅₀ ± SE ¹	95% Fiducial CI ¹	Slope ± SE	p ²	χ ² ³
AMEP20	0.65 ± 0.10 × 10 ⁸ a	0.41 – 0.97 × 10 ⁸	0.34 ± 0.03	<0.001	2.53
AMEP43	1.50 ± 0.29 × 10 ⁸ b	1.00 – 2.19 × 10 ⁸	0.38 ± 0.03	<0.001	3.67
AMEP84	1.47 ± 0.34 × 10 ⁸ b	0.93 – 2.33 × 10 ⁸	0.32 ± 0.10	<0.001	3.37

¹ Median lethal concentration (LC₅₀) and fiducial confidence intervals (CI) are in conidia per milliliter of suspension; SE—standard error of the mean; LC₅₀ values sharing the same letter are not significantly different ($\alpha = 0.05$, Tukey's HSD test); ² p-value for a slope from the regression analysis; ³ Pearson χ^2 goodness-of-fit test on the probit model ($\alpha = 0.05$, df = 2); Mean control mortality of adults was 2.15 ± 0.22% in the bioassays.

A general pattern of mycosis progress in treated groups of weevils is depicted by mean cumulative mortality in Figures 2 and 3. Dynamics of disease development in tested populations depended on conidia concentrations. The first mortality due to fungal infection occurred on 6 and 12 days post-treatment (dpt) when treated with the highest and lowest conidia concentrations, respectively. At the highest conidia concentration, the mean daily mortality during 13–18 dpt (1.81% ± 0.10%) was significantly higher ($p < 0.01$) than for 1–12 dpt (0.34% ± 0.03%) or higher ($p > 0.05$) than for 19–21 dpt (1.48% ± 0.17%) in the bioassay ($F_{(2,63)} = 45.77$, $p < 0.01$). Generally, mean daily mortality for all isolates increased until a culmination on 16–18 dpt and then gradually decreased to the end of the experiment. Development of mycosis induced by the three most pathogenic strains (AMEP20, AMEP43, and NREP84) had a similar trend, but mortality culminated two to three days earlier reaching its maximum on the 14–16 days post-treatment, which was followed by a gradual decrease in counts of killed weevils.

Figure 2. Mean cumulative mortality data (%) with standard errors of *Hylobius abietis* adults after their inoculation with conidia suspensions of *Beauveria* strains at concentrations of 1 × 10⁶ conidia mL⁻¹ (A) and 1 × 10⁷ conidia mL⁻¹ (B).

Figure 3. Mean cumulative mortality data (%) with standard errors of *Hylobius abietis* adults after their inoculation with conidia suspensions of *Beauveria* strains at concentrations of 1×10^8 conidia mL⁻¹ (A) and 1×10^9 conidia mL⁻¹ (B).

Out of the strains included in the pathogenicity tests, AMEP20 *B. bassiana* strain demonstrated the greatest biological activity against *H. abietis* adults, and mycosis by the strain developed faster in the test populations. As many as $64.00\% \pm 2.87\%$ weevils succumbed to infection by the strain at the highest conidia concentration (1×10^9 conidia mL⁻¹) until day 15, when mortality culmination was observed. Total cumulative mortality ($83.25\% \pm 3.17\%$) induced by this strain at conidia concentration of 1×10^9 conidia mL⁻¹ was significantly higher than the mortality of the other two highly virulent strains ($F_{(2,12)} = 17.62$, $p < 0.01$), namely AMEP43 ($75.33\% \pm 3.59\%$) and NREP84 ($72.00\% \pm 4.12\%$). The same trend was documented for all tested conidia concentrations. Based on the above results, AMEP20 *B. bassiana* strain was selected for the feeding damage bioassay and the outdoor experiment.

3.3. Feeding Damage Bioassay

Treatment of weevils with *B. bassiana* inoculum affected their feeding damage on the bark of Scots pine twigs. *B. bassiana*-treated weevils damaged smaller area of bark per day than untreated individuals and mean daily damaged area of bark decreased with increasing conidia concentrations used for weevils treatment (Figure 4). The effect of *B. bassiana* on damage increased with time and was more prominent, although not significant ($p > 0.05$), at higher ($\geq 1 \times 10^7$ conidia mL⁻¹) conidia concentrations. While total eaten area of bark per weevil was 197.83 mm^2 for untreated weevils and $197.21\text{--}227.44 \text{ mm}^2$ for *B. bassiana*-treated weevils when evaluated 11 dpt, the consumed area per weevil for a period of 33–46 dpt dropped to 157.25 mm^2 in the untreated group and to $0\text{--}88.42 \text{ mm}^2$ for *B. bassiana*-treated individuals. No significant difference in feeding damage was observed between males and females either in the untreated control ($F_{(1,4)} = 0.05$, $p = 0.84$) or after inoculating with conidia suspensions ($F_{(1,4)} = 0.07$, $p = 0.81$ for 1×10^5 conidia mL⁻¹; $F_{(1,4)} = 0.05$, $p = 0.83$ for 1×10^6 conidia mL⁻¹;

$F_{(1,4)} = 0.02, p = 0.91$ for 1×10^7 conidia mL^{-1} ; $F_{(1,4)} = 0.08, p = 0.80$ for 1×10^8 conidia mL^{-1}). Mortality of weevils due to *B. bassiana* in the feeding damage bioassay increased with conidia concentration and time (Figure 5). No mortality was detected 11 days after treatment with any conidia concentrations. At the end of the bioassay (46 dpt), mean cumulative mortality varied from 38.89% to 100% depending on the concentration of conidia. Mean cumulative mortality in the control reached 4.44%, but mycosis by *B. bassiana* was not confirmed in the cadavers.

Figure 4. Mean daily damaged area (mm^2) of bark on twigs by *Hylobius abietis* adults after their inoculation with different conidia suspensions of *Beauveria bassiana* strain AMEP20. Values above bars represent numbers of surviving *H. abietis* adults on specific days post-treatment.

Figure 5. Mean cumulative mortality data (%) with standard errors of *Hylobius abietis* adults from the feeding damage bioassay after their inoculation with conidia suspensions of *Beauveria bassiana* strain AMEP20.

3.4. Outdoor Bioassay with *B. bassiana* Treatment

In the outdoor experiment, AMEP20 *B. bassiana* strain killed weevils that had been either treated with conidia suspensions (a direct treatment) or had been allowed to feed on the saplings previously treated with the fungal inoculum (an indirect treatment) (Table 4). Total mortality of weevils after the direct conidia treatment reached 85% or 100% with confirmed *B. bassiana* mycosis of 65% or 85% when

evaluated 32 or 46 days post-treatment, respectively. *B. bassiana* infection recorded in groups of weevils exposed to the conidia-treated saplings varied from 30.0% to 76.5% after 32 days of incubation time and from 55.0% to 88.2% after 46 days. The conidia applied on the saplings remained active during at least six days post-treatment, what was demonstrated by a mycosis development in the exposed groups of weevils. The highest level of mycosis was recorded in the group of weevils exposed to those saplings that were treated with conidia two days before. In this group of weevils, the proportion of mycosed individuals was even higher (76.5% or 88.2% after 32 or 46 days of incubation time, respectively) than in the group directly treated with conidia suspension (65.0% or 85.0% after 32 or 46 days of incubation time, respectively), but the difference was not significant ($p > 0.05$). No mycosed weevils were detected on the untreated control saplings.

Table 4. Mortality of *H. abietis* adults after their direct and indirect treatment with conidia of the most virulent *Beauveria bassiana* strain AMEP20 in outdoor experiment.

Incubation Time		Control	Direct Treatment	Indirect Treatment—Exposure of Adults to Spruce Saplings 0–6 Days Post-Treatment (dpt)						
				0 dpt	1 dpt	2 dpt	3 dpt	4 dpt	5 dpt	6 dpt
32 days	Total mortality	0%	85.0%	70.0%	82.4%	88.2%	70.6%	45.0%	65.0%	52.6%
	Mycosis	0%	65.0%	60.0%	64.7%	76.5%	52.9%	30.0%	55.0%	31.6%
	χ^2 statistic ¹			0.107	0.001	0.579	0.554	4.912	0.417	4.356
	<i>p</i> -value			0.744	0.985	0.447	0.457	0.027	0.519	0.037
46 days	Total mortality	0%	100.0%	85.0%	100.0%	100.0%	94.1%	70.0%	75.0%	73.7%
	Mycosis	0%	85.0%	75.0%	82.4%	88.2%	76.5%	55.0%	75.0%	63.2%
	χ^2 statistic ¹			0.625	0.047	0.082	0.436	4.286	0.625	1.762
	<i>p</i> -value			0.429	0.828	0.774	0.509	0.038	0.429	0.184

¹ Chi-square tests performed to determine whether there was a significant difference between the proportion of mycosed adults in direct and indirect treatments ($\alpha = 0.05$).

4. Discussion

EPF are important natural control agents of insects and have been a subject of an intense study since the end of the nineteenth century. More than 700 fungal species in 100 orders are estimated [43]. A great majority of perspective fungi for mass production and use in biocontrol are those from the Hypocreales (Ascomycota) order [38]. Until now, the occurrence of EPF in *H. abietis* populations has been poorly studied, in spite of the importance of this forest pest. From fungal pathogens of large pine weevil documented in previous studies, hypocrealean EPF, especially those of *Beauveria* genus, have been the most common [35,44,45]. Current investigations on the occurrence of EPF in field populations of *H. abietis* in Slovakia showed that *Beauveria* infection is consistently present but at a low prevalence level (0–5.97%). This corresponds with results of other studies. For example, in Ireland a percentage of weevils infected with *Beauveria* sp. ranged from 1.3% to 3.5% [32]. In Europe, three *Beauveria* species: *B. bassiana*, *B. brongniartii* (Sacc.) Petch and *B. caledonica* Bissett and Widden, have been recorded from field populations of *H. abietis* to date. While *B. bassiana* was identified in this survey, we did not identify *B. brongniartii* and *B. caledonica* from any of collected individuals. On the other hand, we detected *B. pseudobassiana* that is the first record of this pathogenic fungus on *H. abietis*. Altogether, six in vitro isolates of this species were obtained from the cadavers and thus the fungus cannot be considered a rare EPF in large pine weevil populations. Recent analyses of soil samples in Slovakia indicated that *B. pseudobassiana* preferred forest habitats over field crops or meadows [62] assuming its better adaptation to forest ecosystems. On the other hand, this species was not identified from forest soil samples in Poland [63]. Since virulence tests ranked two strains of *B. pseudobassiana* (AMEP43 and NREP84) among strains possessing high efficacy against the weevils, the pathogenic potential of this fungus against forest insect pests deserves a further study. *B. brongniartii* is a well-known pathogen of soil inhabiting coleopteran larvae, but was also identified from larvae of *H. abietis* in Austria [35]. Its pathogenicity to *H. abietis* has also been demonstrated in laboratory experiments [49]. *B. caledonica* is a species originally described from moorland soil in Scotland [64] and was also found as

a naturally-occurring pathogen of *H. abietis* and other curculionids in the northern UK, Ireland, Poland, Austria, France, Slovakia, and New Zealand [32,35,45,65,66]. Results of laboratory assays suggest that *B. caledonica* has a potential to be a biocontrol agent of curculionids. Bioassays with *B. caledonica* strains from Slovakia and New Zealand demonstrated their high pathogenicity to adults of *Ips typographus* (L.) (Curculionidae: Scolytinae) and other scolytid beetles [45,66]. Although pathogenicity of fungi from another hypocrealean genus, *Metarhizium*, to *H. abietis* was demonstrated in laboratory and field experiments [36,50,67], this fungus was not found naturally infecting weevils in this survey or in other studies [32,35].

Now, it is generally accepted that a traditional morphotaxonomic determination of *Beauveria* species is not sufficient and an application of molecular methods is required. Most records on *B. bassiana* and *B. brongniartii* made before the comprehensive multigene phylogenetic analysis and taxonomic revision of *Beauveria* genus [54,55] must be judged with caution. Both taxa were complexes of species consisting of morphologically similar but cryptic lineages. Following the novel molecular approach in *Beauveria* taxonomy, several new species have been described [55,68–74]. Up until now, five species (*B. bassiana*, *B. brongniartii*, *B. caledonica*, *B. pseudobassiana*, and *B. varroae* S.A. Rehner and Humber) have been documented in Europe. While *B. bassiana*, *B. brongniartii*, and *B. pseudobassiana* are globally distributed soilborne entomopathogens with a broad host-range occurring in a variety of habitats, *B. varroae* is known only from ectoparasitic mites of honeybee in France, although it can infect also coleopteran hosts [55]. Because the previous studies on EPF diversity in field-collected large pine weevil in Europe have been based on the morphotaxonomic principles, some *Beauveria* species might have been misidentified or remained undetermined.

As shown in the current field surveys, *Beauveria* infection is one of natural factors of *H. abietis* mortality. Although natural epizootics are not typical for these fungi, laboratory and field experiments have already shown their efficacy against this forest pest [32,36,50,67]. Previous research on testing *Beauveria* spp. against *H. abietis* produced optimistic outcomes, but effective biocontrol methods using these fungi are still lacking. It is generally known that a selection of highly virulent strains is a prerequisite for successful implementation of EPF in the biocontrol of insect pests. A series of our laboratory bioassays showed that all tested *Beauveria* strains could infect adults of *H. abietis* and sporulate on cadavers. No effect of sex of tested weevils was detected on the susceptibility against the fungal strains. To our knowledge, *B. pseudobassiana* has not been tested against *H. abietis* yet, and the current virulence tests bring optimistic results. Based on the interspecific variability in pathogenicity of the strains we cannot conclude that any of tested *Beauveria* species demonstrated better results in the biological properties than the other ones. Two highly virulent *B. pseudobassiana* strains and one *B. bassiana* strain were identified in the virulence bioassays. *B. bassiana* strain AMEP20 was selected for further investigation due to higher mortality, and thus lower values of LC₅₀, than mortality obtained after application of *B. pseudobassiana* strains.

Generally, disease development was relatively slow in tested weevils. The most virulent strain (AMEP20) caused 29.97% cumulative mortality on day 12 and 83.25% mortality on day 21, when the highest conidia concentration (1×10^9 conidia mL⁻¹) was tested. In a similar study by Ansari and Butt [36], *B. bassiana* strains caused 53% mortality of *H. abietis* on day 12, if the weevils were treated by submersion into conidia suspension (1×10^8 conidia mL⁻¹). In this study, the mortality caused by AMEP20 strain and a concentration of 1×10^8 conidia mL⁻¹ was 63.27% on day 21, but it was only 16.65% on day 12. Ansari and Butt [36] explain the prolonged time to the host death by the thick and tough cuticle of adults that can form a barrier against the entomopathogenic fungi. This slow killing rate by entomopathogenic fungi might be a limitation factor for their effective use against adults of the large pine weevil. It was documented that three adults can completely strip the bark of a spruce sapling within six days [75]. On the other hand, *H. abietis* adults can live for up to four years [1,76] and females lay eggs each season (from May to September) during their lifetime. Considering this fact, the slow action of fungi does not seem to be such an important factor. Any measure applied to kill adults can effectively contribute to the control of weevil population. Due to the slow action of *B. bassiana* during the

virulence bioassays, we also tested an effect of treatment by conidia suspensions (including sublethal concentrations) on the feeding damage by adult weevils. Treated weevils damaged significantly smaller area of bark than untreated individuals. The effect was observed for all tested conidia concentrations and was more prominent when mycosis progressed. The results clearly demonstrate that although the infected individuals lived relatively long after inoculation, their feeding on twigs was reduced resulting in smaller damage in comparison with healthy weevils. Spruce trees are the main host plants of *H. abietis* in Slovakia, but we used *P. sylvestris* twigs for the feeding damage experiment because this food was preferred by adult weevils when compared to *P. abies* [60]. There is a general agreement in several studies that optimum temperature for feeding of adult weevils is 20 °C [60,77,78] (Christiansen and Bakke, 1968; Christiansen, 1971; Leather et al., 1994). At this temperature a weevil consumed on average 252.9 mm² of *P. sylvestris* bark during 7 days [60]. In the current bioassay, an untreated (control) weevil consumed less food (197.83 mm²) during 11 days, and the food consumption moderately decreased towards the end of the bioassay. We do not know the reasons for the discrepancy with the previous study, but it might be related to the different quality of provided food or a different body size of adults in the studies. There is a considerable variation in the body size within natural populations of *H. abietis* and the weevil size was shown an important factor affecting a feeding rate [79].

Laboratory bioassays are usually carried out under optimal conditions for pathogenesis, which can be very different from environmental conditions in the field. Therefore, efficacy of the selected *B. bassiana* strain (AMEP20) was tested in outdoor conditions. It is well known that environmental factors can significantly reduce viability of *B. bassiana* inoculum when applied to the plant surface. The sunlight belongs to the most limiting factors [80–82]. Viability of conidia decreased to a half after a two-hour irradiation by simulated sunlight in laboratory [83,84]. Significant variability in conidia susceptibility to simulated sunlight exists among *B. bassiana* strains [85]. The conidia viability may also vary depending on the plants, on which the inoculum had been applied. For example, viability of *B. bassiana* conidia was high up to 26 days on lettuce and celery leaves [86], but it significantly decreased on *Medicago sativa* L. and *Agropyron cristatum* (M. Bieb.) P. Beauv. plants after 16 days [87] and on soya plants after 10 days [79]. In the current study, the *B. bassiana* conidia were active on spruce plants 6 days after its application and caused high mortality of *H. abietis*. The results also confirm that a contact of weevils with plants treated with inoculum leads to their high mortality. A similar study showed that a sustained contact of weevils with *B. bassiana*-treated spruce bark caused high infection rates [48]. The outcomes indicate that the application of conidia suspension on saplings could be a perspective approach in the biocontrol of *H. abietis*.

5. Conclusions

The current study investigated natural prevalence of EPF in populations of *H. abietis* adults and pathogenicity of local *Beauveria* strains against *H. abietis* adults with the goal to identify those possessing a high potential for biocontrol of this pest preferably in vulnerable forest areas, e.g., national parks, nature reserves and other areas with restrictions applied in the conventional pest management. Two *Beauveria* species (*B. bassiana* and *B. pseudobassiana*) were active in natural populations of large pine weevil. The laboratory bioassays suggested that AMEP20 *B. bassiana* strain could provide an effective and an acceptable level of *H. abietis* control in laboratory and simulated field conditions. Additional studies under actual field conditions are currently being conducted to evaluate the performance of the strain in forests. In addition, horizontal transmission of infection in the host populations, inoculum formulation and its introduction into *H. abietis* populations are also studied.

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