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Original Paper

Entomopathogenic fungi associated with *Stomoxys calcitrans* in Slovakia and efficacy of local fungal strains against the stable fly

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The stable fly, *Stomoxys calcitrans*, is the most important ectoparasite of livestock throughout the world. Entomopathogenic fungi are natural antagonists of stable flies with the potential to be used as biocontrol agents in integrated management programmes of this pest. The prevalence and species diversity of entomopathogenic fungi in stable fly populations were documented on a dairy cattle farm in south-western Slovakia. The prevalence of fungal infection was low and the maximum percentage of infected flies reached 2.72% during May–October in 2016. Three *Beauveria* species were identified from infected flies, *B. bassiana*, *B. pseudobassiana* and *B. brongniartii*. *Beauveria pseudobassiana* and *B. brongniartii* are reported from *S. calcitrans* for the first time. In laboratory tests, the pathogenicity of four entomopathogenic fungi *B. bassiana*, *B. pseudobassiana*, *M. anisopliae* and *Isaria fumosorosea* against adult stable flies was compared. The flies were susceptible to infection by the fungi and the most virulent isolate of *B. bassiana* (IKEP10) was selected for spore formulation testing and field trials. We tested three types of spore formulations and the aqueous spore suspension was more effective against stable flies than spores formulated in the powder or oil suspension. Repeated treatments of the interior of the stable with *B. bassiana* spores at fortnightly intervals during June–August resulted in a 10 times higher prevalence of fungal infection in the stable fly population compared with the untreated stable, but the treatments did not effectively reduce the fly population. The selected *B. bassiana* isolate may represent an interesting tool in the biocontrol of stable fly populations, but further experiments are needed to design an appropriate formulation and enhance its efficacy.

Keywords: biological control, entomopathogenic fungi, formulation, pathogenicity, stable fly

1 Introduction

The stable fly, *Stomoxys calcitrans* L. (Diptera, Muscidae), is a serious economic pest of confined and pastured livestock throughout the world (Foil and Hogsette, 1994). It causes stress and discomfort to animals due to its biting, blood-feeding and ability to transmit various pathogens (Patra et al., 2018). The agitated animals react to the fly attacks by stamping their feet, flinging their heads, twitching their skin and switching their tail. To prevent being bitten, they tend to congregate in groups, which might result in heat stress and reduced feeding (Mullens et al., 2006). Because of the consistent fly feeding, there is less weight increase and lactation, which has a direct economic consequence. Mean infestation levels by stable flies on cattle can reach 2–4 individuals per leg (Taylor et al., 2012), but even up to 700 flies per animal have been documented (Solórzano et al., 2015). It was reported that more than three stable flies per leg could result in economic damage (Hogsette et al., 1987; Taylor et al., 2012). In 2009, the estimated annual loss of milk production per cow associated with stable flies ranged from 42 to 299 kg and economic losses reached 13–85 USD per cow and year in the United States (Taylor et al., 2012).

Stable fly biology is well understood. Saprophagous larvae live in the moist decomposing vegetable matter (such as decaying hay, silage or compost) that has been mixed with animal excrement and urine. Manure by itself is not an appropriate substrate for their development unless it is combined with plant biomass (Taylor et al., 2012; Cook et al., 2018). Larval development passes through three instars and can take anything from eight

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days (at 26 °C) to several months (during winter). Pupal development takes place inside the puparium directly in the substrate. Adult flies are obligate hematophages and feed on the blood of warm-blooded animals. They prefer to feed on the lower regions of the front legs mostly during the early morning or late evening (Hogsette et al., 1987; Foil & Hogsette, 1994). Females require an average of 1.8 feedings per day and numerous blood meals to complete ovarian development. Males feed 2.8 times per day on average (Harris et al., 1974). Eggs are placed in clusters of 20–100 within decaying vegetable matter and a single female can generate 60–800 eggs during the lifetime (Foil & Hogsette, 1994). A whole life cycle can be completed in as little as three weeks in optimal conditions. Among environmental factors, rainfall and temperature have a substantial impact on the population dynamics of stable flies (Taylor et al., 2012; Semelbauer et al., 2018).

Management options to control stable fly populations include sanitation (i.e. removal of potential developmental sites), insecticide or repellent treatments targeted either directly on animals or on resting and developmental sites, insecticide-impregnated ear tags, various trapping systems to catch adult flies, as well as biological control using parasitoid wasps (e.g. *Spalangia* spp., *Muscidifurax* spp.), entomopathogenic bacteria, fungi or nematodes (e.g. *Bacillus thuringiensis* Berliner, *Metarhizium anisopliae* (Metschn.) Sorokīn, *Steinernema* spp., *Heterohabditis* spp.) (e.g. Hogsette et al., 1987; Meyer et al., 1990; Watson et al., 1995; Thomas et al., 1996; Cook, 2020; Taylor, 2021). Generally, insect pests are targeted for control in such life phases when they are immobile, aggregated and accessible. The most appropriate time is during the larval stage for most dipteran pests. On the other hand, stable fly larval development substrates are diverse, dispersed, and difficult to locate. Furthermore, developmental sites are frequently transient, as substrates are only favourable for fly growth during specific stages of decomposition (Taylor & Berkebile, 2006; Talley et al., 2009). As a result, it is difficult to effectively direct treatments against the larvae and most control efforts are focused on the adult stage (Taylor, 2021).

The insecticide resistance development and environmental safety issues considerably limit the use of insecticides for stable fly population management, necessitating the employment of environmentally friendly techniques. Entomopathogenic fungi, as an alternative to insecticides, could be a promising approach. Entomopathogenic fungi are a phylogenetically diverse group of eukaryotic and heterotrophic microorganisms pathogenic to insects that grow a part of their life cycle inside the host body (Lacey et al., 2015). There are currently about 750 species of entomopathogenic fungi classified into approximately 100 genera, with the majority of significant species belonging to Ascomycota (Hypocreales) and Entomophthoromycota (Entomophthorales). The fungi produce sexual and/or asexual spores that germinate following contact with hosts, hyphae penetrate the cuticle of hosts and invade the haemocoel. When hosts die, hyphae emerge to cover the cadaver and produce spores, which infect new hosts. Entomophthorales cause frequent epizootics in insect populations (including fly pathogens, e.g. *Entomophthora muscae* (Cohn) Fresen.), but they are difficult to mass-produce for targeted application. Instead, Hypocreales, which are found in low prevalence levels in insect populations, are simple to produce on a mass scale. The hypocrealean fungi are considered promising candidates for microbial control of various insect pests (Lacey et al., 2015) and several fungi are commercially available in the sale as microbial control agents, for example *Beauveria bassiana* (Bals.-Criv.) Vuill., *B. brongniartii* (Sacc.) Petch, *M. anisopliae*, *Lecanicillium lecanii* (Zimm.) Zare & W. Gams and *Isaria fumosorosea* Wize, (de Faria et al., 2007; Reddy et al., 2013). *Metarhizium brunneum* Petch, *M. anisopliae*, *L. lecanii*, and *B. bassiana* are among the hypocrealean entomopathogenic fungi that infect stable flies (Cook, 2020). Several studies have used *B. bassiana* and *M. anisopliae* strains against larval and/or adult stages of stable flies. Up to 90% mortality was found in adult stable flies exposed to the fungi (Watson et al., 1995; Moraes et al., 2010; López-Sánchez et al., 2012; Weeks et al., 2017).

Species richness of entomopathogenic fungi parasitising stable flies has not yet been determined in Slovakia, hence the goal of this study was to identify species diversity of hypocrealean fungi infecting adults of *S. calcitrans* in cattle stables. We also evaluated the efficacy of local isolates of four entomopathogenic fungi against adult stable flies in the laboratory and naturally infested cow stables.

2 Material and methods

2.1 Study site

Stable flies were collected and field experiments were undertaken in the dairy cattle production farm – PD Šenkvice – in the village of Šenkvice, south-western Slovakia. The farm is located (48° 18′ 10″ N, 17° 21′ 34″ E, altitude 177–178 m a. s. l.) on the foothills of the Little Carpathian Mountains and the climate in this area is characterized by warm and dry summers (mean July temperatures are 18–21 °C) and mild winters (mean January temperatures vary from -1 to -2 °C). The mean annual precipitation of 500 mm is distributed unevenly throughout the year. Most of the rain falls during spring

Figure 1 Area of the farm PD Šenkvice and the arrangement of cattle stables (the buildings highlighted in red) indicating the position of stables used in the field study (the stable D – treated with *Beauveria bassiana* spores, the stable B – treated with water without spores)
Source: www.zbgis.skgeodesy.sk (© GKÚ, NLC; 2017–2019)

and autumn. The farm consists of six cattle stables arranged in parallel in a south-eastern (entrance) to the north-western (exit) direction (Figure 1). Internal stable dimensions are approximately 70 × 10 m and each stable is designed to house 56 cows in different stages of the milking and reproductive cycle. Two stables (Figure 1, stables C and D), neighbouring each other and situated in the middle of the complex of six stables, were selected to collect stable flies and assess the prevalence of entomopathogenic fungi in 2016. Stables B and D were used for the application of *B. bassiana* spores in 2017.

2.2 Insect collecting

Adult flies were collected by sweep-netting using a standard-size net with a 45 cm hoop and 1.5 m handle. Sampling events were always conducted on sunny days between the hours of 10 a.m. and 1 p.m. when stable fly activity was at its peak. Flies were collected inside and outside the stables.

Flies collected inside the stables were used to evaluate the natural prevalence of fungal infection in 2016 (May–October) and the prevalence of fungal infection after application of *B. bassiana* spores in 2017. The sweep-netting took around 60 min and involved 15 sweep-net samples per stable. In this scenario, five sweeps of the net equalled one independent sweep-net sample. To evaluate the prevalence of mycosis, the collected flies (unsexed) from all sweep-net samples from a given stable and date were mixed in a cage (300 × 300 × 600 mm wire frame covered with fine monofilament polyester fabric) and incubated for seven days at 25 ±2 °C, 80% relative humidity and a 12 h/12 h (L/D) photoperiod. Dead flies were removed from cages and deposited separately into individual wells of a 96-well plate (Brand GmbH & Co KG, Germany) on a daily basis. The plates were covered with moistened filter paper and kept at 25 ±2 °C for an additional seven days to promote the development of mycosis. Cadavers with symptoms of mycosis were stored at 4 °C until pathogens were identified.

Flies caught outside the stables, usually resting on the stable walls exposed to the sun, were intended for laboratory treatments. In this case, the sweep-netting continued until we had collected enough individuals for bioassays. The collected flies were maintained in cages as

described above at 16 ±2 °C, 60% relative humidity and in the dark until they were employed in bioassays the next day.

2.3 Identification of entomopathogenic fungi

Entomopathogenic fungi infecting stable fly adults were identified microscopically (500×) according to morphological characteristics of microstructures (Humber, 2012). The morphological identification was coupled with a sequencing study of the internal transcribed spacer (ITS) region of ribosomal DNA (ITS1-5.8S-ITS2) and a partial gene of the translation elongation factor (TEF-1α). DNA was extracted from the fungal biomass (mycelium with spores) taken from the surface of dead flies using DNeasy Plant Mini Kit (Qiagen, Crawley, West Sussex, UK) according to the producer's instructions. The selected DNA regions were amplified with the primer pairs ITS5/ITS4 for ITS and 983F/2218R for TEF-1α. The PCR products were purified by ExoI/FastAP (Life technologies, CA, USA) and Sanger sequenced using Macrogen service (Macrogen Europe B.V., Amsterdam, the Netherlands). The sequences were compared to DNA sequences in the NCBI GenBank sequence database using the Blast-N algorithm.

2.4 Fungal cultures

Five different isolates of *B. bassiana*, *B. pseudobassiana* S.A. Rehner & R.A. Humber, *I. fumosorosea* and *M. anisopliae* were tested in this study (Table 1). The isolates were obtained either from naturally infected *S. calcitrans* in Šenkvice or from a fungal culture collection of the Institute of Forest Ecology of Slovak Academy of Sciences (Nitra, Slovakia). The isolates from the fugal culture collection were selected based on their high efficacy against insect pests in previous studies (Schemmer et al., 2016; Barta et al., 2018). Each isolate was passaged twice through *S. calcitrans* adults and then re-isolated onto Sabouraud Dextrose Agar (SDA) plates before laboratory bioassays. The fungal spores were produced by incubating the cultures on SDA plates at 25 ±1 °C and constant illumination for two weeks. Spore biomass harvested by a sterile spatula from the cultures was layered in a glass Petri dish lid and air-dried overnight inside a laminar flow cabinet. The viability of spores was assessed by germination tests on agar plates after a 12 h incubation at 25 ±1 °C and only spores with viability higher than 95% were used in the following bioassays.

2.5 Laboratory bioassay 1: pathogenicity tests

The five fungal isolates were tested for pathogenicity against stable fly adults in the laboratory. Adult flies were anesthetised by cold treatment at 4 °C for 3 minutes and divided into groups of 20 individuals in 250 ml disposable transparent plastic cups (95 mm in diameter). The cups with 20 flies were turned upside-down and placed on Petri dish lids lined with a 10 cm disc of Whatman No. 1 filter paper treated with 3 ml of spore suspension of the fungal cultures. The spore suspensions were prepared by suspending the spores in 0.01% (w/v) Tween® 80 and using the Neubauer haemocytometer the spore concentration was adjusted to 1×10^6 spores ml^{-1}. The spore-treated filter paper disc was left in the dish lid for 60 min and then was replaced with a sterilised filter paper disc without spores. Sucrose water (10% w/v) soaked cotton balls were provided to the flies *ad libitum* in the cups during the bioassay. The cups with flies were held within a 35-L transparent polypropylene box (580 × 380 × 175 mm) at constant illumination and temperature of 25 ±2 °C. The box was lined with wet filter paper and closed with a latching lid to maintain saturated humidity inside. Dead flies were counted and removed from the cups daily for seven days. The cadavers were washed in sterilised water and placed into individual wells of a 96-well plate (Brand GmbH & Co KG, Germany). The plates were covered with moistened filter paper and incubated at 25 ±2 °C for additional seven days to promote the development of mycosis. Only cadavers with symptoms of mycosis were used to estimate pathogenicity. The experiment was run in a completely randomized design with five replicates for each fungal isolate, including controls treated with sterilised water instead of spore suspension. A total of 100 flies were tested for each fungal isolate and the control.

Table 1 A list of fungal isolates used in the laboratory bioassays against adults of *Stomoxys calcitrans*

Isolate	Fungus species	Origin of isolate (host, locality and isolation date)
IKEP10	*Beauveria bassiana*	*Ips typographus* L.; Michalovo, Slovakia; April 2012
NREP63	*Beauveria bassiana*	*Stomoxys calcitrans* L.; Šenkvice, Slovakia; June 2016
NREP64	*Beauveria pseudobassiana*	*Stomoxys calcitrans* L.; Šenkvice, Slovakia; July 2016
AMEP03	*Isaria fumosorosea*	*Cameraria ohridella* Deschka & Dimić; Veľký Cetín, Slovakia; May 2015
AMSO09	*Metarhizium anisopliae*	Soil; Žemberovce, Slovakia; June 2009

2.6 Laboratory bioassay 2: spore formulation tests

The most pathogenic fungal isolate of *B. bassiana* (IKEP10) selected in the pathogenicity tests was used to evaluate the efficacy of three types of spore formulations in laboratory treatments. Powder formulation, aqueous suspension and oil suspension of spores were tested. The fungal spores were produced as mentioned above. The powder formulation was prepared by mixing the *B. bassiana* spores (1.5 g) with 100 g of skimmed milk powder (Merck KGaA, Germany) and the concentration of spores in the formulation was measured by a germination test on an agar plate. A final concentration of 1×10^9 CFU g^{-1} (colony forming units per gram of formulation) was achieved by adding an aliquot of skimmed milk powder to the mixture. The aqueous spore suspension was prepared by suspending the *B. bassiana* spores in 0.01% (w/v) TWEEN® 80 and the concentration was adjusted to 0.34×10^9 spores ml^{-1}. The oil spore suspension was prepared by suspending the spores in a mixture of white medicinal oil and Exxsol™ DSP 30/75S (ExxonMobil, Belgium) (blended in a ratio of 1 : 4) to achieve a concentration of 0.34×10^9 spores ml^{-1}.

Cohorts of 30 stable fly adults were isolated separately in 250 ml disposable transparent plastic cups. As described above, the cups with flies were turned upside-down and placed on Petri dish lids lined with a 10 cm disc of Whatman No. 1 filter paper treated with either 3 ml of the aqueous or oil spore suspensions or 1 g of the powder formulation. The flies were exposed to the treated filter paper for four different times: 1, 5, 15 and 60 min. After exposure, the Petri dish lid and treated filter paper were replaced with a sterilised lid and filter paper. The cups with 30 flies were then placed inside 35-L transparent boxes and were incubated as mentioned above. The experiment was conducted in a completely randomized design with five replicates per each combination of exposure time and spore formulation. In the controls, the filter paper discs were treated with spore-free formulation. A total of 150 flies were tested for each combination of exposure time with spore formulation and also for the control.

2.7 Field trial

In 2017, the stable D (Figure 1) was treated with an aqueous suspension of *B. bassiana* spores (isolate IKEP10) and the stable B was treated with deionised water. The spore suspension (1×10^8 spores ml^{-1}) was prepared as described above. A single wall (70 × 2 m) of the stables was evenly treated either with 10 L of spore suspension (in the stable D) or 10 L of deionised water (in the stable B) using a backpack atomiser. The treatments were carried out seven times at fortnightly intervals from May 31 to August 23. The prevalence of mycosis in fly populations was estimated from samples collected by sweep-netting, which started on June 14 and continued until October 11 approximately every fortnight. The fly samples were collected inside the stables and were monitored for infection in laboratory conditions as described above (see 2.2).

2.8 Data analysis

Mortality data from the pathogenicity tests were corrected for natural (control) mortality using Schneider-Orelli's formula and were subjected to ANOVA. Before ANOVA, the mortality data were tested for normality (Shapiro-Wilk test) and then arcsine-transformed $\left(n' = \arcsin\sqrt{n}\right)$ to obtain a normally distributed data set. The *post hoc* Tukey's HSD test was performed to separate and compare means if significant differences ($p = 0.05$) were detected. Cumulative mortality data of flies at different exposure times of the formulation tests were subjected to probit analysis and a median effective exposure time to get 50% mortality (MET50) was evaluated. Values of MET50 were subjected to ANOVA and the *post hoc* Tukey's HSD test. A Chi-square test was used to compare the proportion of infected flies between treated and control stables during the field trial. Counts of stable flies caught by sweep-netting in the treated and control stables were subjected to Student's t-test to test the null hypothesis that counts of flies in the treated and untreated stable are not different. All analyses were conducted using Minitab 17 (© 2013 Minitab Inc.).

3 Results and discussion

3.1 Entomopathogenic fungi associated with stable fly populations

In 2016, as many as 2650 flies were caught in the farm PD Šenkvice from May 25 to October 26 and 28 dead individuals (1.06%) displaying external symptoms of fungal infection were observed. The infected flies occurred on each sampling date, except for two sampling events in September and October (Table 2). Fungi from the infected flies were preliminarily identified as *Beauveria* sp. by microscopic examination and the Blast-N analysis of DNA sequences of partial ITS and TEF-1α regions revealed three *Beauveria* species. The fungi were identified as *B. bassiana* from 21 cadavers, *B. pseudobassiana* from four individuals and *B. brongniartii* from three dead flies. The identification was supported by a high degree (> 99%) of identity with the GenBank sequences of the neotype *B. bassiana* strain (ARSEF 1564) or the type strains of *B. pseudobassiana* (ARSEF 3405) and *B. brongniartii* (ARSEF 617). The prevalence of *Beauveria* infection in the population of stable fly was low and almost even throughout the season with no distinct peak. The percentage of infected individuals varied

Table 2 Counts of *Stomoxys calcitrans* adults caught by sweep-netting and percentage of individuals infected with *Beauveria* spp. in natural populations in the farm PD Šenkvice in 2016

Date	No. of caught flies	Infection prevalence*	Identified fungal species
25 May	144	1.39% (2)	*B. bassiana*
9 June	202	1.49% (3)	*B. bassiana*, *B. brongniartii*
22 June	121	0.83% (1)	*B. bassiana*
5 July	384	1.04% (4)	*B. bassiana*, *B. pseudobassiana*
20 July	104	0.96% (1)	*B. bassiana*
3 August	335	1.19% (4)	*B. bassiana*, *B. pseudobassiana*
18 August	304	0.99% (3)	*B. bassiana*
31 August	229	1.31% (3)	B. bassiana
14 September	184	2.72% (5)	*B. bassiana*, *B. pseudobassiana*
28 September	474	0%	–
13 October	134	0%	–
26 October	179	1.12% (2)	*B. brongniartii*

*numbers in parentheses represent counts of infected flies collected on the particular date

between 0 and 2.72% depending on the collecting date (Table 2). Two *in vitro* cultures were isolated from infected flies (Table 1) and used in laboratory bioassays.

The *Beauveria* genus is a group of entomopathogenic fungi with global distribution and a broad host range including species from many insect orders (e.g. Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, Orthoptera, Thysanoptera and others). To date, 24 *Beauveria* species have been recognised, but not all of them have been reported as parasites of dipteran hosts (e.g. Rehner et al., 2011; Khonsanit et al., 2020). In Europe, as many as five species, *B. bassiana*, *B. brongniartii*, *B. pseudobassiana*, *B. caledonica* Bissett & Widden and *B. varroae* S. A. Rehner & R. A. Humber, have been recorded up to now. The first three species are distributed worldwide in various habitats on many insect hosts. Steinkraus et al. (1990) reported *B. bassiana* infection on a muscoid fly (*Musca domestica* L.) for the first time. Subsequently, this fungus was documented from or tested against the stable fly in several studies (e.g. Watson et al., 1995; Moraes et al., 2010; López-Sánchez et al., 2012; Weeks et al., 2017). *Beauveria pseudobassiana* and *B. brongniartii* are reported from *S. calcitrans* for the first time in this study. This observation is interesting because *B. brongniartii* is mostly known as a parasite of coleopteran larvae living in soil (Zimmermann, 2007) and among hosts of *B. pseudobassiana* dominate mostly species from Coleoptera and Lepidoptera orders (Wang et al., 2020). Moreover, *B. pseudobassiana* is a species probably better adapted to a forest ecosystem since it was recognised to prefer forests over agricultural or meadow habitats (Medo et al., 2016). *Beauveria caledonica*, originally described from the soil in Scotland, has been reported only from coleopteran hosts in Europe or New Zealand (Glare et al., 2008; Barta et al., 2018, 2020). *Beauveria varroae* is originally known from ectoparasitic mites of honeybees in France, but it was also documented from coleopterans and lepidopterans (Rehner et al., 2011; Barta et al., 2020).

The prevalence of *Beauveria* infection in populations of stable flies was low throughout the sampling season, but it corresponds to the biological properties of this group of fungi (Zimmermann, 2007). The enzootic prevalence of entomopathogenic fungi in populations of muscoid flies, including *S. calcitrans*, was also documented in other studies and varied around 1% (e.g. Steinkraus et al., 1990; Skovgård & Steenberg, 2002; Siri et al., 2005). Observations in this study indicate that the fungal pathogens do not possess a natural capacity to significantly suppress stable fly populations. From the viewpoint of stable fly management in farms, a release of mass-produced fungal inoculum into fly populations is necessary to increase the prevalence of the fungi and to suppress populations. Natural enemy augmentation is an effective alternative in suppressing pests when naturally occurring enemies are not sufficiently abundant or effective. Therefore, there have been several efforts for selecting effective fungal strains and developing augmentation techniques for the introduction of fungal inoculum into stable fly populations (Cook, 2020). We tested the pathogenicity of four entomopathogenic fungi against the stable fly to select the most effective one for the treatment of cattle stables.

3.2 Pathogenicity of entomopathogenic fungi to stable fly adults in laboratory

Under laboratory conditions, all tested fungi were pathogenic to stable fly adults (Figure 2), but the mortality rate varied significantly among isolates ($F_{(4,24)}$ = 36.25, p <0.01). *Metarhizium anisopliae*, the isolate AMSO09, was significantly the least pathogenic fungus with the mean mortality rate of 7.20 ±1.36%. The *B. bassiana* isolates, IKEP10 and NREP63, demonstrated the greatest biological activity against the fly with the mean mortality rates of 29.60 ±1.57% and 21.60 ±0.81%, respectively. Mortality attributed to the isolate IKEP10 was significantly (p <0.01) higher compared to the isolate NREP63. There was no significant difference (p >0.05) in pathogenicity between *B. pseudobassiana* (isolate NREP64) and *I. fumosorosea* (isolate AMEP03), however, the *B. pseudobassiana* isolate was more effective in reducing the viability of the fly (15.40 ±0.68% of mean mortality) than the *I. fumosorosea* isolate (11.80 ±1.36% of mean mortality). The mortality rate in the controls was variable, reaching between 0 and 3%, and only mycosis by *B. bassiana* was observed in the cadavers. Based on the pathogenicity bioassay, the adult flies were the most susceptible to isolate IKEP10 and therefore this isolate was selected for the spore formulation tests and the field trial.

The use of entomopathogenic fungi as biocontrol agents against *S. calcitrans* has already been investigated in several studies and virulence results varied significantly among the tested fungal species and isolates (Cook, 2020). Although a high susceptibility of adult flies to entomopathogenic fungi was demonstrated, larvae and pupae were either tolerant or only weakly susceptible to infection (e.g. López-Sánchez et al., 2012; Cruz-Vazquez et al., 2015; Baleba et al., 2021). Even, antifungal activity of *S. calcitrans* larvae against the fungi was documented (Moraes et al., 2014). Therefore, adult flies were included only in the current research. The pathogenicity of tested Slovak isolates was highly variable, but other studies also showed differences in pathogenicity among fungal strains (e.g. López-Sánchez et al., 2012; Weeks et al., 2017). We showed that *S. calcitrans* was significantly more susceptible to *Beauveria* infection than to *Isaria* or *Metarhizium*. Interestingly, in other studies, *M. anisopliae* strains were capable of infecting and causing high mortality of stable flies under *in vitro* conditions (López-Sánchez et al., 2012; Weeks et al., 2017; Baleba et al., 2021) and were selected for field trials to control the stable fly (Cruz-Vazquez et al., 2015). To the best of our knowledge, there are no data in the literature regarding the susceptibility of *S. calcitrans* to *B. pseudobassiana*. However, it should be taken into account that *B. pseudobassiana* is morphologically indistinguishable from *B. bassiana* (Rehner et al., 2011). Therefore, some previous studies that reported pathogenicity of

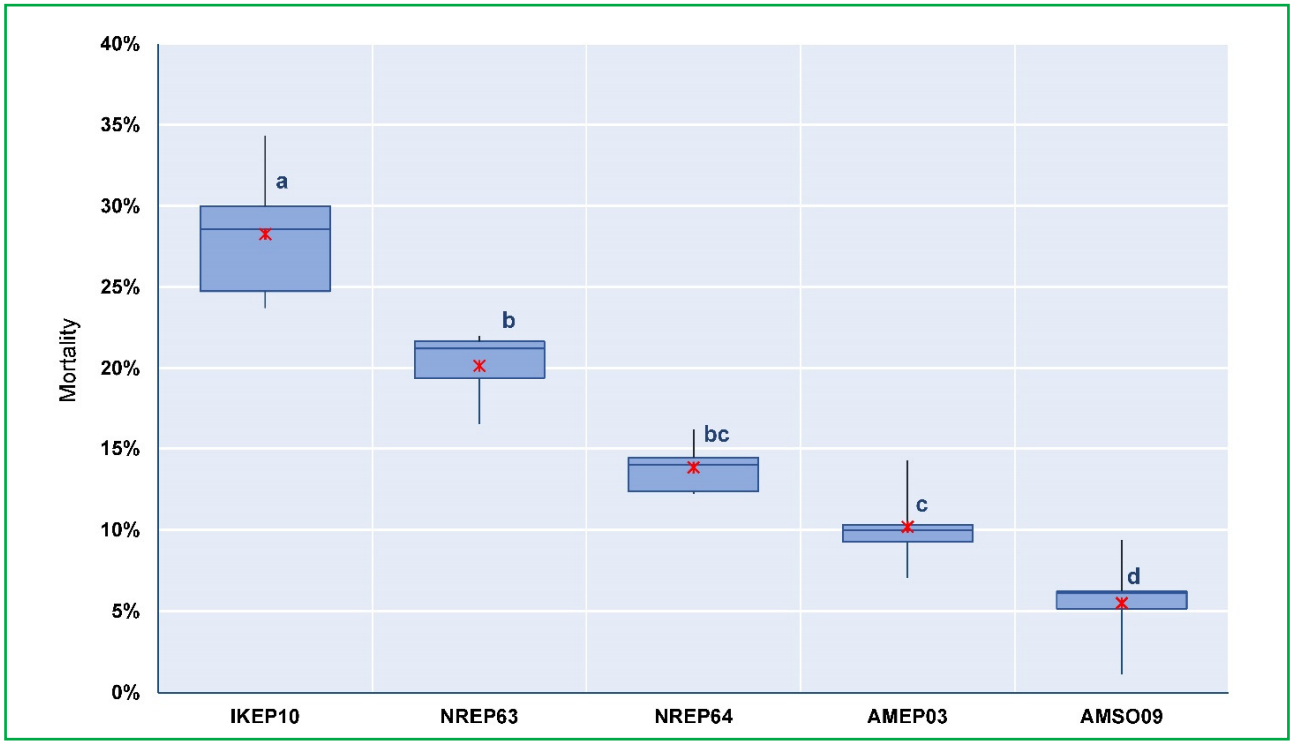

Figure 2 Box and whisker plot showing mortality of *Stomoxys calcitrans* adults treated with spores of five different isolates of entomopathogenic fungi in the laboratory bioassay; mortality values indicated by the same letter are not significantly different (Tukey HSD test; p = 0.05)

morphologically determined *B. bassiana* strains against *S. calcitrans* should not be considered reliable without additional molecular confirmation of fungal identity. In several laboratory bioassays, high virulence against stable flies was reported by entomopathogenic fungi and they were recommended as a prospective biocontrol agent in farms. Up to 90% mortality was demonstrated in adult stable flies exposed to *B. bassiana* and *M. anisopliae* at a dose of 1×10^8 spores ml^{-1} (e.g. Watson et al., 1995; López-Sánchez et al., 2012; Weeks et al., 2017). We report considerably lower mortality of flies (up to 30%) after treatment with the most pathogenic *B. bassiana* isolate (IKEP10), but the isolate can be considered effective because the spore concentration used in the bioassay was 100-fold lower in our study. Moreover, a comparison of the results obtained in this study with those obtained in other studies is difficult due to differences in the methods used.

3.3 Effect of spore formulations on pathogenicity to stable fly adults

Appropriate formulation of fungal inoculum can reduce adverse effects of environmental factors in the field and can significantly influence the efficacy of fungal pesticides. Several trials on the use of different formulations of spores for fly control have been performed and they often produced ambiguous or mixed results (e.g. Barson et al., 1994; Watson et al., 1995; Cruz-Vazquez et al., 2015; Weeks et al., 2017). We tested three types of spore formulations intending to determine compatibility with the most pathogenic *B. bassiana* isolate (IKEP10). The tested formulations demonstrated the efficacy of spores against stable fly adults in the laboratory. The percentage mortality varied between 5.43 ±1.30% and 96.00 ±1.25%, depending on the formulation type and exposure time (Table 3). The aqueous spore suspension was more effective than spores formulated as a powder or an oil suspension and the difference in effectiveness was more pronounced at shorter exposure times. After a one-minute exposure to the spore-treated surface, the mortality of flies attributed to the aqueous spore suspension was significantly higher (20.49 ±1.73%) than mortality rates achieved with either the powder formulation (8.76 ±1.70%) or oil spore suspension (5.43 ±1.30%) ($F_{(2,12)} = 20.16$, $p < 0.001$). The same effect of spore formulation type on mortality rate was observed after a five-minute exposure ($F_{(2,12)} = 10.07$, $p = 0.002$), but not ($p > 0.05$) at longer exposure times (15 and 60 min). The mortality rate increased with exposure time, which made it possible to estimate the relationship between time and morality by probit analysis. The estimated values of median effective time (MET50) varied from 3.81 to 8.10 minutes with probit regression slopes of 0.58–0.71, depending on the spore formulation type (Table 4). Approximately two-fold shorter exposure time was necessary to kill 50% of tested stable flies with the aqueous spore suspension than with the equivalent spore dose (1×10^9 spores per a 10 cm paper disc) applied as either the powder formulation or the oil suspension (Figure 3).

Table 3 Mean mortality of *Stomoxys calcitrans* adults after their exposure to the surface treated with different formulations of *Beauveria bassiana* spores (isolate IKEP10) in laboratory bioassays

Spore formulation	Mean mortality ($\bar{x}$ ±SE) at different exposure times to the treated surface*			
	1 min	5 min	15 min	60 min
Powder formulation	8.76 ±1.70% a	40.46 ±4.05% a	76.32 ±3.29% a	90.00 ±2.36% a
Aqueous suspension	20.49 ±1.73% b	66.50 ±5.67% b	76.01 ±1.55% a	96.00 ±1.25% a
Oil suspension	5.43 ±1.30% a	46.52 ±2.29% a	70.59 ±1.89% a	92.00 ±2.26% a

*the mean mortality values were corrected for the control mortality that ranged between 0 and 3%; the values for particular exposure time followed by the same letters are not significantly different (Tukey HSD test; $p = 0.05$)

Table 4 Results of probit analyses testing the efficacy of *Beauveria bassiana* isolate IKEP10 against *Stomoxys calcitrans* adults after their exposure to the surface treated with different spore formulations

Spore formulation	MET50 (min)*	95% fiducial *CI*	Slope	*p***	χ^{2}***
Powder formulation	7.96 ±0.83 b	6.39 – 9.65	0.68 ±0.05	0.000	2.77
Aqueous suspension	3.81 ±0.51 a	2.83 – 4.85	0.58 ±0.05	0.000	1.25
Oil suspension	8.10 ±0.83 b	6.53 – 9.79	0.71 ±0.05	0.000	3.19
ANOVA****	$F_{(2,12)} = 15.41$, $p < 0.001$				

* mean value ±standard error of means; ** *p*-value of a slope from regression analysis; *** Pearson χ^2 goodness-of-fit test on the probit model ($p = 0.05$, $df = 2$); **** ANOVA statistics testing for differences in the lethal times between the spore formulations; mean values followed by the same letter in the row are not significantly different
mortality in the controls ranged between 0 and 3% in the assays

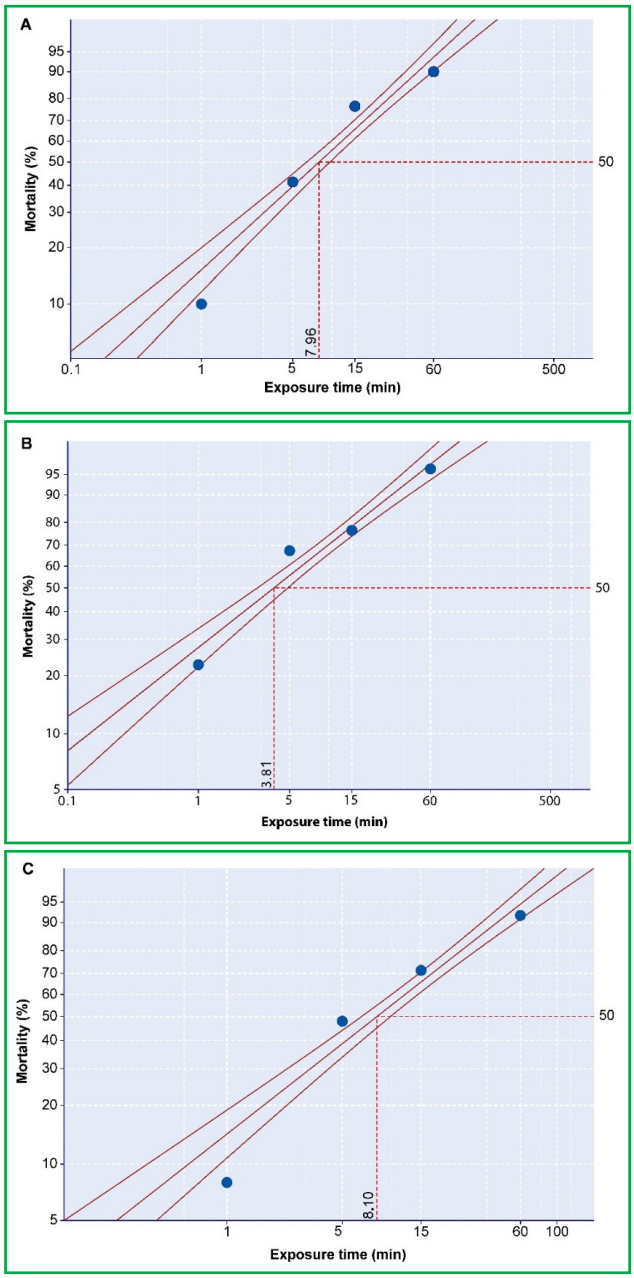

Figure 3 Linear regressions from the probit analyses showing the relationship between mortality of *Stomoxys calcitrans* and exposure time to the surface treated with different formulations of *Beauveria bassiana* spores
A – powder formulation, B – aqueous suspension of spores and C – oil suspension of spores

In our study, the mineral oil-based formulation of *B. bassiana* spores showed the least efficacy against the fly. Interestingly, other studies demonstrated opposite results. Vegetable oils (linseed and soybean oils) increased the efficacy of *M. anisopliae* spores against house fly two-fold compared with aqueous spore suspension (Barson et al., 1994). Oil formulations of *M. anisopliae* spores increased virulence against insects and improved the shelf life of spores and resistance to adverse environmental stresses during field application (e.g. Malsam et al., 2002). Former studies also showed that dust formulation of *B. bassiana* spores was more effective for controlling *M. domestica* and *S. calcitrans* than aqueous formulations (Watson et al., 1995) what does not correspond to our observations. The aqueous spore suspension of the Slovak *B. bassiana* isolate was significantly more effective, especially after the short contact of flies with the treated surface. We also believe that this formulation is more convenient for application in stables than powder or oil formulations. Stable flies tend to rest on walls and ceilings of buildings during the day and thus such areas seem to be appropriate for treatment with the fungal spores. However, powder formulations do not adhere to vertical surfaces very well and potential inhalation of formulation powdery particles, including spores, by animals and workers must be also taken into consideration. Allergic reactions could occur in workers who are exposed repeatedly to high concentrations of spores during their production or when they are applied in an inhalable form (Zimmermann, 2007).

3.4 Treatment of stables with *B. bassiana* spores

Aqueous spore suspension of *B. bassiana* isolate IKEP10 was used for the treatment of cattle stable because it was significantly more effective than the oil spore suspension or powder formulation in the laboratory bioassay. Repeated treatments of the stable interior with *B. bassiana* spores in fortnightly intervals resulted in a higher prevalence of fungal infection in the stable fly population compared with the untreated (control) stable (Table 5). The mycosis prevalence varied from 3.33 to 15.60% in the treated stable and reached up to 1.99% in the control stable. The highest mortality rate due to fungal infection was observed on August 23 and it continuously decreased after this date until the end of monitoring on October 11. The decrease in infection rate from the end of August was because the application of spores did not continue after August. Anyway, the prevalence of fungal infection in the treated stable was significantly (p <0.05) higher when compared with the control stable. Although the prevalence of mycosis was higher in treated stable, the difference in abundance of flies between the treated and untreated stables was not significant (t = 1.41, 16 df, p = 0.17). This can be explained by the high flight activity of flies and a short distance between treated and

Table 5 Counts of *Stomoxys calcitrans* adults caught by sweep-netting and percentage of individuals with *Beauveria* infection in stables treated and non-treated with spores of *Beauveria bassiana* (isolate IKEP10) in the farm PD Šenkvice in 2017

Date	No. of caught flies in stables		Infection prevalence*		χ2 test** (*df* = 1)
	treated	control	treated	control	
14 June	220	175	6.81% (15)	0%	–
28 June	195	154	11.79% (23)	0%	–
11 July	256	222	8.59% (22)	0.90% (2)	13.43, p <0.05
26 July	252	201	10.71% (27)	1.99% (4)	11.77, p <0.05
9 August	235	214	11.06% (26)	0.93% (2)	17.46, p <0.05
23 August	218	188	15.60% (34)	1.59% (3)	20.17, p <0.05
9 September	237	254	10.55% (25)	1.18% (3)	15.44, p <0.05
27 September	221	205	5.43% (12)	0.98% (2)	6.23, p >0.05
11 October	150	187	3.33% (5)	0%	–

*numbers in parentheses represent counts of infected flies; **Chi-square test was used to compare the proportion of infected flies between treated and control stables

untreated stables (approximately 20 m). Thus flies could move from untreated to treated stables. It was also noted that the distribution of stable flies among and within stables was uneven on this farm (Semelbauer et al., 2018). Many studies showed that the stable fly is susceptible to entomopathogenic fungi under laboratory conditions (e.g. Watson et al., 1995; Moraes et al., 2010; López-Sánchez et al., 2012), but there are only a few reports on the efficacy of fungi under natural infestation conditions. For example, aqueous spore formulation of *M. anisopliae* applied directly on cows reduced natural infestation by *S. calcitrans* up to 73% (Cruz-Vazquez et al., 2015). Watson et al. (1996) showed that *B. bassiana* helped control house flies after the application of conidia to calf bedding. We assume that if all stables in the farm had been treated, the total abundance of flies could have been reduced. To ensure sufficient fungal efficacy, mass-release of spores should start at the beginning of the fly season (i.e. early May) and should be repeated during the summer due to the relatively short viability of fungal spores under field conditions. Secondary effects of entomopathogenic fungi on flies by inhibiting oviposition and infecting eggs have also been documented (Moraes et al., 2010, 2014; Baleba et al., 2021), which may also contribute to the reduction of population growth.

4 Conclusions

Three fungal species, *B. bassiana*, *B. pseudobassiana*, and *B. brongniartii*, infected *S. calcitrans* populations at a low prevalence level in the research site, and the fungi had a limited capacity to naturally suppress the abundance of flies. The pathogenicity of five isolates of entomopathogenic fungi was evaluated against stable fly adults under laboratory conditions and the most virulent *B. bassiana* isolate IKEP10 was selected for the field trials. Aqueous spore suspension of IKEP10 isolate was the most effective formulation against the adult flies. Although the isolate was pathogenic to the flies when applied to the stable walls, and the mycosis prevalence increased up to 15.60% in the population, no effect on the fly abundance was observed. Laboratory bioassays showed that *B. bassiana* may be a good option for inclusion in the integrated pest management programmes for stable flies. However, further research is needed to confirm the potential of *B. bassiana* in the field and to develop appropriate spore formulations to improve its efficacy as a mycoinsecticide.

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