

Pine defoliator *Bupalus piniaria* (L.) (Lepidoptera: Geometridae) and its entomopathogenic fungi.

2. Pathogenicity of *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria farinosa*

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Fungi *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria farinosa* (local strains), were tested on 3rd and 4th instar larvae of pine defoliator Bordered White moth (Pine Looper), *Bupalus piniaria* (L.) (Lepidoptera: Geometridae) under laboratory conditions. Reared larvae were sprayed with the fungal suspension at a concentration of 1×10^8 conidia mL⁻¹. All three fungi species tested increased mortality of larvae as compared to control (distilled water spray). The highest (100%) mortality was caused by *B. bassiana* within 12 days, followed by *M. anisopliae*: 18 days were needed to reach the same mortality level. The lowest mortality was caused by *I. farinosa*: even double spray resulted only in $56.67 \pm 6.6\%$ mortality. The most effective entomopathogene was *B. bassiana*.

Key words: biological control, Bordered White (Pine Looper), *Bupalus piniaria* (L.), forest pests, insect pathogens, mortality, microscopic fungi

INTRODUCTION

Entomopathogenic fungi provide ecological services as they are involved in natural control of pest populations. Bordered White moth, *Bupalus piniaria* (L.) (Lepidoptera: Geometridae), is an autumnal defoliator of pine forests spread around the world over all range of pine stand (Šmits et al., 2008; Straw et al., 2002). The interest in *B. piniaria* control was caused by outbreaks in European countries around the Baltic Sea, (e. g. Sweden and Finland (Långström et al., 2004), Latvia (Šmits et al., 2001; Zarinš, 2001), Poland (Borowski, 2007) as well as Russia (Meshkova, 2002), and the UK (Barbour, 1980) leading to defoliations of thousands hectares

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of pine forest. In Lithuania, *B. piniaria* activity has been reported in the centre of outbreaks caused either by Pine-tree Lappet moth, *Dendrolimus pini* L. (Lepidoptera: Lasiocampidae) or Pine Beauty moth, *Panolis flammea* Schiff. (Lepidoptera: Noctuidae) (Valenta et al., 2004; Vasiliauskas, 2010).

Modern pest management strategies are aimed at decreasing the usage of chemicals with a negative environmental impact and high cost. Moreover, insecticide application close to water bodies and populated areas is strictly limited. An alternative could be a biological means for pest control including microbial preparations. The most biological preparation produced, are based on either bacterium (*Bacillus thuringiensis*) or baculoviruses (Lewis, 1989; Zarinš, 2001; Glowacka, Malinowski, 2006; Hitchman et al., 2007;

Jankevica et al., 2008). For pest control, the environmentally friendly entomopathogenic fungi could be used, as well. Preparations of entomopathogenic fungi, however, constitute a small percentage of the total insecticide market (Yatin et al., 2006), though they are still among the most practical biological control agents for agricultural and forest pest control.

As far as we know, there is no information on fungi application for the Bordered White moth management. Data on *B. piniaria*-associated entomopathogenic fungi as well as on virulence of two species was published recently (Pečiulytė et al., 2010). The most common fungi species associated with *B. piniaria* were those from the genera *Aspergillus* P. Micheli ex Link, *Cladosporium* Link, *Fusarium* Link, *Penicillium* Link, *Mucor* Fresen, *Beauveria* Vuill., *Entomophthora* Fresen., *Lecanicillium* W. Gams & Zareand, and *Paecilomyces* Bainier (Pečiulytė et al., 2010). However, the bioassay carried out under laboratory conditions, revealed low virulence of the most frequently isolated fungi *Fusarium solani* (mart.) Sacc. and *Lecanicillium psalliotae* (Treschew) Zare & W. Gams to *B. piniaria* (Pečiulytė et al., 2010).

The aim of the present study was to evaluate the virulence of well known entomopathogenic fungi to *Bupalus piniaria* larvae under laboratory conditions. Cosmopolitan entomopathogenic fungi *Beauveria bassiana* (Bals.-Criv.) Vuill., *Metarhizium anisopliae* ((Metschnikov) Sorokin and *Isaria farinosa* (Holmsk.) Fr. [= *Paecilomyces farinosus* (Holmsk.) A. H. S. Brown & G. Smith], were tested.

METHODS AND MATERIALS

Insects

Pupae of Bordered White moth, *Bupalus piniaria* (L.) (Lepidoptera: Geometridae), were collected under litter of pine stand in southern Lithuania (Druskininkai district) in late autumn of 2007. For overwintering pupae were kept under conditions close to natural. In spring emerged adult moths were transferred into cages for mating and supplied with Pine twigs (*Pinus sylvestris* L.) for egg laying. Hatched larvae were fed with fresh needles on 10–15 cm long pine twigs for feeding and were kept at 19–22 °C temperature. The twigs were collected in nature from different trees and replaced every 2 days regularly.

Fungi

Entomopathogenic fungi used in the tests belong to phylum Ascomycota: *Beauveria bassiana* (Bals.) Vuill. [family Cordipitaceae, order Hypocreales, class Sordariomycetes], *Metarhizium anisopliae* (Metschnikov) Sorokin [family Clavicipitaceae, order Hypocreales, class Sordariomycetes], and *Isaria farinosa* (Holmsk.) Fr. [syn. *Paecilomyces farinosus* (Holmsk.) A. H. S. Brown & G. Smith] [family Trichocomaceae, order Eurotiales, class Eurotiomycetes] classification following Sung et al. (2007). Fungal strains used in the study were obtained from Fungi Culture Collection (Biodeterioration Research Laboratory, Institute of Botany, Nature Rese-

arch Center, Vilnius, Lithuania), maintained for a long-term storage. The strains originated from soil samples collected in Lithuania. Sporulation peculiarities, enzymatic (chitinase, protease and amylase) activity and virulence of the fungal isolates were examined annually. Three strains (*B. bassiana* DPK-07-b-8, *Metarhizium anisopliae* DPK-08-m-3 and *I. farinosa* DPK-06-f-11) were chosen for the present study, based on some biochemical characteristics (not presented) as well as on pathogenicity, revealed for larvae and adults of Indian meal moth, *Plodia interpunctella* (Lepidoptera: Pyralidae) (Būda, Pečiulytė, 2008) and larvae of Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) (personal communication, Pečiulytė, 2010).

To produce the conidia, fungi were grown on quarter strength Sabouraud dextrose agar (SDA, Liofilchem, Italy) for 14–21 hours at 25 ± 2 °C in the dark. The viability of matured conidia was checked before use for bioassay. Germination of conidia was evaluated by plating 10 µl aliquots (five replicates) of the suspension (10 conidia per mL) on 2.2% water agar medium. The conidial suspension was spread on the medium with a sterile spatula and incubated for 17–24 h at 25 ± 2 °C. Three samples were cut from the agar medium with germinating conidia, each was placed on a slide, and 200 conidia were counted. A conidium was deemed as germinated when the length of the germ tube exceeded a half size of the conidia. When the percentage of germinated conidia reached ≥92.7–98.2%, mature fungal culture was used for bioassay. Usually, conidia of the grown and matured cultures of the fungi remained viable for two to three months; therefore the culture could be used for the preparation of conidial suspensions repeatedly as inoculums to continue the tests within this period.

Conidia for bioassay tests were collected with a spatula from 14–20 d old cultures and placed in sterile tubes containing 10 mL of sterile distilled water (dH₂O). Conidia were washed twice with aliquots of sterilized dH₂O; each time passing through 2 layers of cheesecloth to remove any large particles and hyphae. Conidia were finally suspended in dH₂O, placed in a sonicating water-bath (MSE) at 15 °C for 20 min to prevent clumping. Based on a hematocytometer counting, the conidial suspension was adjusted to obtain 1 × 10⁸ germinating conidia mL⁻¹ dH₂O concentration, chosen for testing, following the recommendations (Freimoser et al., 2003; Zimmermann, 2008) as well as based on our earlier experience (Pečiulytė et al., 2010).

Bioassay

In the laboratory, the 3rd and 4th instars of *B. piniaria* larvae were carefully transferred from the rearing chambers and placed into sterile Petri dishes (10 larvae per dish). The larvae were sprayed either with one mL of the 1 × 10⁸ germinating conidia mL⁻¹ of appropriate fungus suspension or with the same amount of dH₂O (control). Sprayed larvae were transferred on pine twigs. Twigs were soaked and installed in glass cages (in volume 750 mL). Pine twigs, containing

both mature and current-year shoots, were used. Mortality of *B. piniaria* larvae was recorded daily.

Larvae were sprayed with *B. bassiana* and *M. anisopliae* suspension once and with that of *I. farinosa* twice (with 7 days interval). After 7 days of incubation, the larvae treated with *I. farinosa* conidia were divided into two groups and were sprayed repeatedly: one group with the fungus suspension, and the other with dH₂O (control). Each treatment was conducted in triplicate. Larvae were reared for three weeks at 20 ± 1 °C, >65% relative humidity and 12 : 12 light : dark (L : D) photoperiod. The mortality percentage was counted daily. Larvae cadavers were removed from the cages, and their surface was sterilized in 5% sodium hypochlorite, then in 75% ethanol solution, rinsed in plenty of sterile distilled water and left to dry for 48 h. After that procedure, cadavers were placed on sterile moistened filter paper disks in Petri dishes and incubated at room temperature in clean desiccators. Fungal grown cadavers were regarded as killed by fungus, while the rest were regarded as dead for other reasons. The fungi grown on cadavers were observed under a microscope (×4–7, ×180 or ×720), then were isolated in pure culture on Potato Dextrose Agar (PDA, Liofilchem, Italy) medium to confirm fungus assignment to the species. Re-identification was made basing on the micro-morphology and using the keys as indicated above. Slide cultures were used for microscopic examination of intact fungal reproductive structures. The slides were prepared in the same way as for conidi-

al germination analysis, except water-agar was changed to malt extract agar (MEA, Liofilchem, Italy). Sporulating mycelium was transferred from the cadaver on the medium layer on a slide. The slide cultures were incubated at 24 °C in a humid chamber and allowed to grow until reproductive structures appeared (for 4–7 days).

Statistical analysis

All the calculations (means and standard error) were performed using MS Excel. Standard error was estimated for every experimental point and marked in Fig. 1 and 2 as error bars. Mortality data were adjusted for control mortality. The median lethal time period LT₅₀ (lethal time to reach 50% mortality of the treated insects) was determined using probit analysis (Sokal, Rohlf, 1995).

RESULTS

Effect of *Beauveria bassiana* and *Metarhizium anisopliae*

Fungal isolates of both species caused significantly higher mortality of *B. piniaria* larvae as compared to control. Dynamics of the mortality differed depending on the fungi tested. During the seven-day period after using fungal isolates, higher larval mortality caused *B. bassiana* DPK-02-d comparing to *M. anisopliae* DPK-06-d (40.1 ± 3.6% and 8.9 ± 1.8%, respectively) (Fig. 1). Larvae mortality dynamics in *B. bassiana* DPK-02-d and *M. anisopliae* DPK-06-d tests during the eighteen-day period suggested a marked difference

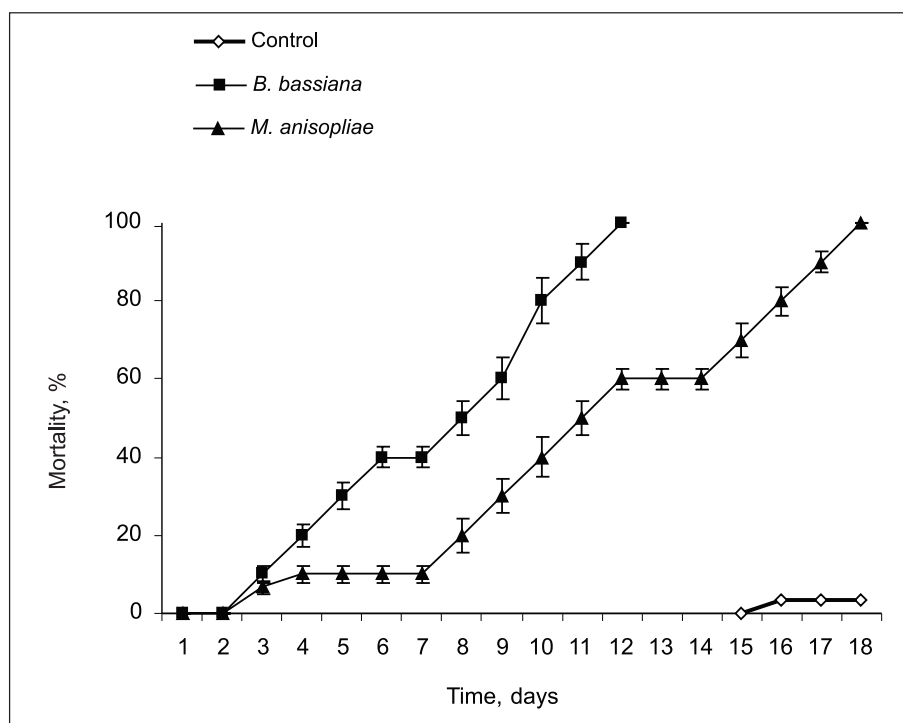


Fig. 1. Mortality dynamics of *Bupalus piniaria* larvae following application of *Beauveria bassiana* DPK-02-d and *Metarhizium anisopliae* DPK-06-d at the concentration of 1×10^8 germinating conidia/mL⁻¹. In the control, larvae were sprayed with sterile distilled water. Vertical bars represent standard error

in the virulence. Significant effect of *B. bassiana* was revealed already within the first days following spray as larvae mortality reached $41.4 \pm 3.6\%$ after 5 days (Fig. 1). The time period required to cause 50 % larvae mortality (median lethal time, LT_{50}) caused by *B. bassiana* was 8 days (Table), while that caused by *M. Anisopliae* was 11 days. The period when 100% mortality (LT_{100}) was reached also differed depending on the fungus applied: 12 and 18 days for *B. bassiana* and *M. Anisopliae*, respectively (Table). Plateaus in the dynamics of the larvae mortality was noticed in the *M. anisopliae* DPK-06-d test during the incubation periods from 4th to the 7th and from 12th to 14th day after the spraying. In the control, the very first cadavers were observed only within the 15th day.

Effect of *Isaria farinosa*

The virulence of *I. farinosa* DPK-06-f-11 to *B. piniaria* larvae was remarkably lower than that of the other fungi tested. The dynamics of larval mortality following spraying by *I. farinosa* is presented in Fig. 2.

The larvae were susceptible to fungus *I. farinosa*, however, the median lethal period, required to cause 50% larval mortality (median lethal time, LT_{50}) was recorded only following the second spray with fungus conidia and was equal to 8 days. An extra spray reduced the survival of *B. piniaria* larvae rapidly. During the 7-day period (from 1st to 7th day after spraying) the larval mortality in the test (20.0%) did not differ from that in the control (19.1%). The larvae mortality sharply increased to $56.67 \pm 6.6\%$ after the second spray (at the 7th day) only, it should be noted that mortality remained at the same level till the end of the test (up to 20th day).

Analysis of cadavers

B. piniaria larvae of different instars as well as adult and pupa are presented in Fig. 3. The characteristics of a colony and reproduction structures of fungi tested and appearance of the appropriate fungus on the cadavers are illustrated in the Fig. 4. Healthy larvae are blue-green with two white stripes on each side of the body (Fig. 3: 2).

Table 1. LT_{50} and LT_{100} mortality of *Bupalus piniaria* larvae sprayed with 1×10^8 fungus / mL vu of the culture

Fungal strains tested	Effect	
	LT_{50} (days)	LT_{100} (days)
<i>Beauveria bassiana</i> DPK-02-d	8.0 ± 1.5	11.5 ± 2.0
<i>Metarhizium anisopliae</i> DPK-06-d	10.5 ± 0.5	18.0 ± 1.5

* Significance of LT_{100} at the 0.05 probability level.

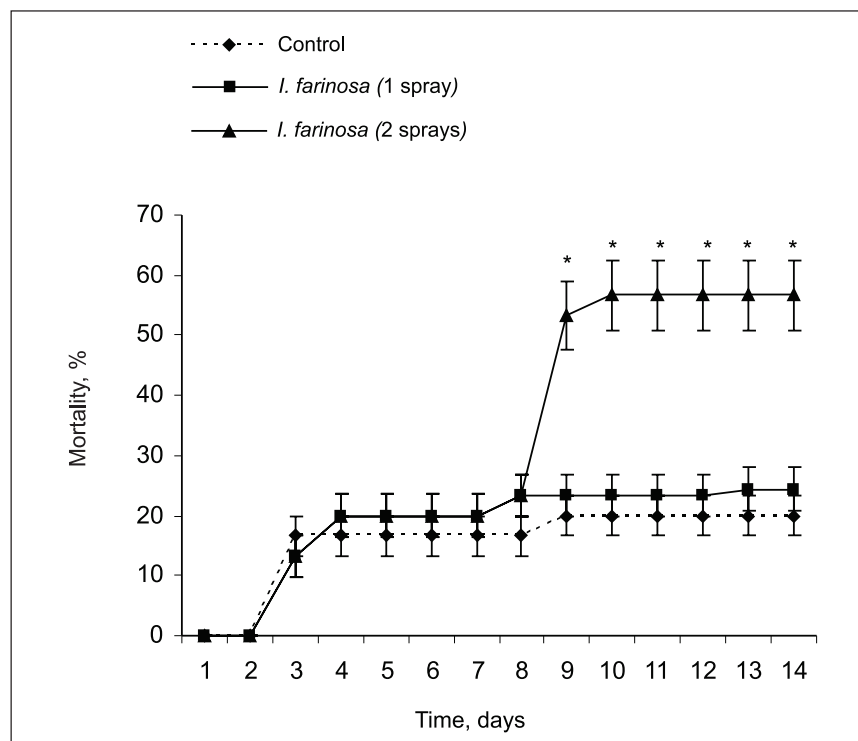


Fig. 2. Mortality dynamics of *Bupalus piniaria* larvae following treatment by *Isaria farinosa* DPK-06-f-11 at the concentration of 1×10^8 germinating conidia ml^{-1} (test group was split after 7 days on first application and one group was sprayed 2nd time resulting insignificant increase of mortality). Control – sterile distilled water spray. Vertical bars represent standard error. Asterisks (*) – statistically significant difference in mortality at the 0.01 probability level

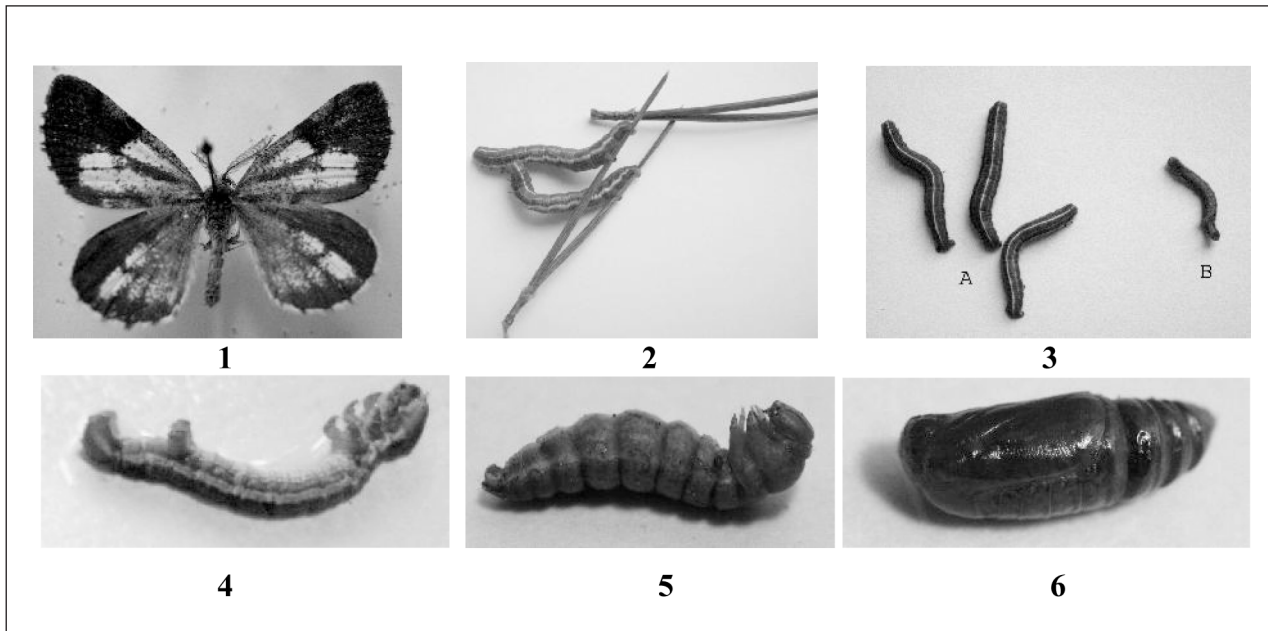


Fig. 3. Bordered White moth (Pine Looper), *Bupalus piniaria* (L.) (*Lepidoptera: Geometridae*): 1 – adult moth; 2 – reared in the laboratory larvae before the test (B); 3 – larvae after 14 days incubation in the control (A) and in the fungus *B. bassiana* test (B); 4 – cadaver of the larvae (*B. bassiana* test) with primary hyphae on it; 5 – mature larvae just before pupation; 6 – pupa

In Fig. 4 the photos show macro- and micro-morphology of the tested fungi after 7 days growth on potato dextrose agar (PDA), and *B. piniaria* larval cadavers at 5 days post-fungal infection in the a moist chamber:

A 1 – *Beauveria bassiana* colony, growing on PDA, A 2 and A 3 – conidiophores consisting of whorls and dense clusters of conidiogenous cells, A 4 – solitary conidiogenous cell and detached conidia, A5 – white fungal hyphae spreading on the outer surface of the cadaver. These hyphae completely covered the cadavers in 5 days.

B 1 – *Metarhizium anisopliae* colony, growing on PDA, B 2 and B 3 – conidiogenous structures and phialides arranged in a candle-like fashion, B 4 – solitary conidiophore and cylindrical conidia, B 5 – columns of the aggregated conidia, B 7 – long conidial chains in the conidial columns, B 6 and B 8 – green layer of the conidiophores in compact patches on the outer surface of the larval cadaver (black drops appear as a result of inducing the prophenoloxidase system activity that causes melanisation of the internal cavity).

C 1 – *Isaria farinosa* colony, growing on PDA, C 2 – conidiophores bearing whorls of divergent branches with phialides and conidia, C 3 – dry divergent chains of conidia on phialides, C 4 and C 5 – white fungal hyphae spreading on the outer surface of the larval cadaver.

Dead larvae were found either fixed to the needles of the pine twig or lying on the bottom of the incubation cage. Infected by fungi larvae almost did not differ from the cadavers transferred from the control cages: cadavers turned grey-green, without any mycelium net, except slight differences in the size of cadavers (cadavers infected

by fungi tended to be smaller than those from the control cages) (Fig. 3: 3, A and B). The cadavers of the larvae, placed in a moist chamber appeared distended and rigid, and later began to darken. Mycelia emerged from dead larvae after complete tissue destruction and depletion of nutrients (Fig. 3: 4, and Fig. 4: C-4, C-5). Mycelium, conidiophores, conidia typical to appropriate fungus grew abundantly on the symptomatic cadavers after 1–3 days of incubation in a moist chamber. Larvae were often covered by dense growth of the fungus in advanced stages of infection. Additional conidiophores were formed singly or in groups near the end of this period. The cadavers turned white, slightly pinkish-white, or green depending on the intensity of fungus overgrowth and fungus species (Fig. 4: B-7, B-8, C-4 and C-5). The abundant conidia of mycelia could be easily scraped from the larvae surface using a sterile loop. Due to fungus development, cadavers began to shrink and sometimes pale-yellow drops of exudates (Fig. 4: B-7 and B-8) appeared. Many larval cadavers infected by *M. anisopliae* crumbled to small pieces. Disease symptoms and analysis of cadavers suggested that the larvae of *B. piniaria* were attacked by tested fungi, and survival was reduced namely due to fungal infection. Percent of the cadavers, which death was caused by the tested fungus, comprised 100% in either of *B. bassiana* and *M. anisopliae* tests, while death of 92% of larvae was caused by the tested fungus when *I. farinosa* was sprayed. The most of cadavers from the control cages and those belonging to the remaining 8% in the *I. farinosa* test were infected by bacteria and only few cadavers by fungi from *Penicillium* Link and *Mucor* Fresen genera.

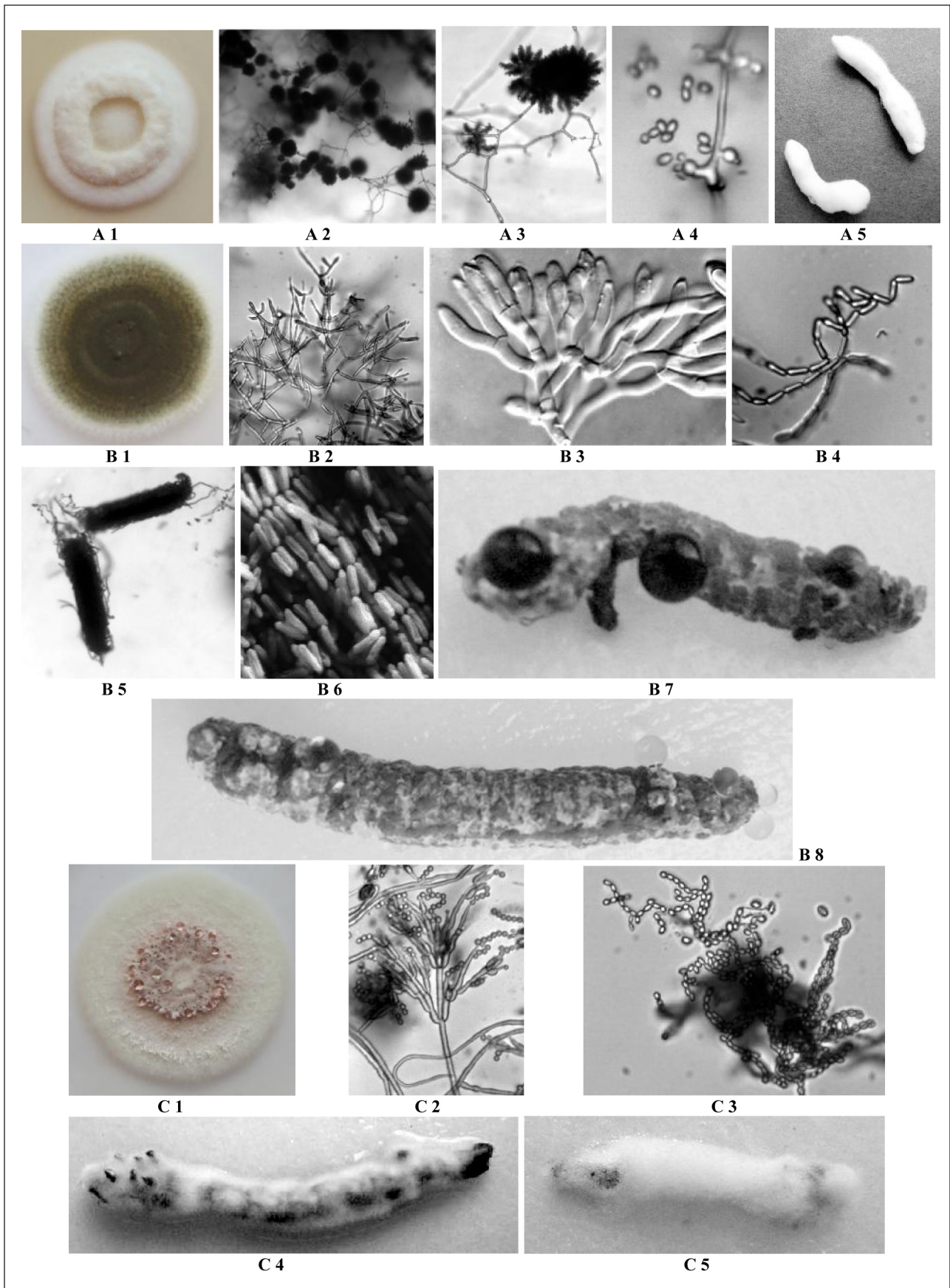


Fig. 4. Photographs showing macro- and micro-morphology of the tested fungi after 7 days growth on potato dextrose agar (PDA), and *B. piniaria* larval cadavers at 5 days post-fungal infection in the a moist chamber

DISCUSSION

Interest in the application of entomopathogenic fungi for insect pest control increases (e. g. Shah, Pell, 2003; Charnley, Collins, 2007). The most widespread insect pathogenic fungi belong to the genera from the order Hypocreales of the phylum Ascomycota (viz, *Beauveria* Vuill., *Lecanicillium* W. Gams & Zare, *Metarhizium* Sorokin, and *Isaria* Holmsk) (Shah, Pell, 2003; Sung et al., 2007). Highly effective in controlling insects from different classes are fungi species *Beauveria bassiana* (Bals.) Vuill., *Metarhizium anisopliae* (Metschnikov) Sorokin and many isolates of the genus *Isaria* (syn. *Paecilomyces*) Bainier (Humber, Hansen, 2005). However, no information is available on the effect of the fungi on *B. piniaria*. This is the first report of laboratory treatment of *B. piniaria* larvae with the entomopathogenic fungi *B. bassiana*, *M. anisopliae*, and *I. farinosa* (syn. *P. farinosus*), characterised by a wide host range, high viability and virulence of the conidia, i. e. by peculiarities suitable for the effective biological control (Hicks, Watt, 2001; Sung et al., 2007; Charnley, Collins, 2007). Lifestyle of the fungi is specific, as they are facultative saprophytes and can exist either as a plant endophytes and / or form an association with plant roots (White et al., 2002).

In the present study, only *B. bassiana* strain DPK-02-d and *M. anisopliae* strain DPK-06-d were recorded as highly pathogenic to larvae, while *I. farinosa* strain DPK-06-f-11 was much less pathogenic. It should be noted that *B. bassiana* is virulent to the other pine defoliator Pine Beauty moth, *Panolis flammea* (Lepidoptera: Noctuidae) as well (Hicks, Watt, 2000). *B. bassiana* is among the best studied fungi species from Cordycipitaceae family and is used to control a variety of agricultural pests, including whiteflies, beetles, grasshoppers and psyllids (Humber, Hansen, 2005), ticks and mosquitoes (Blanford et al., 2005; Kirkland et al., 2004; Scholte et al., 2005). Virulence of this species is high due to some special peculiarities. Conidia of *B. bassiana* do not require an opening or ingestion and are able to penetrate after attachment on the cuticle surface anywhere (although preferential sites of infection for some insects have been described) (Cho et al., 2006). Rod-shaped aerial conidia of *B. bassiana* facilitate adhesion to the cuticle (Holder, Keyhani, 2005). Analysis of *B. bassiana* aerial conidia revealed a hydrophobin to be the most highly represented transcript alongside with antioxidant proteins (Cho et al., 2006). Hydrophobins are small molecular mass fungal proteins participating in adhesion and pathogenesis (Linder et al., 2005; Wosten, 2001). Antioxidant proteins have been implicated in providing protection against host oxidative defence systems and are important components mediating fungal virulence (Cho et al., 2006).

The other fungal species *M. anisopliae*, formerly known as *Entomophthora anisopliae*, is a widely distributed soil-inhabiting fungus, and has been reported to infect approximately 200 species of insects (Charnley, Collins, 2007). The species, as well as *B. bassiana*, has a broad host range,

though a considerable specificity occurs among individual isolates (Charnley, Collins, 2007). In our study, *B. bassiana* (strain DPK-02-d) was more pathogenic to *B. piniaria* larvae as compared to *M. anisopliae* (strain DPK-06-d): median mortality time (LT_{50} 8 and 11 days, respectively), the period for 100% mortality of the larvae achieving (12 and 18 days), suggests that *B. bassiana* could be more effective in *B. piniaria* control.

I. farinosa is important for the ecological services as well. The use of the species in biocontrol experiments against insect pests under laboratory and field conditions was summarised by Zimmermann (2008). However, in our test among the three fungal strains tested, *I. farinosa* was the least virulent as the mortality of the Bordered White larvae during 4-day period (from 3rd to 7th day after spraying) remained at the level of 20%. Only the second application of the fungus, increased larval mortality to $56.67 \pm 6.6\%$, and this level remained stable up to the 20th day (till the end of the experiment).

CONCLUSION

Three entomopathogenic fungi, *Beauveria bassiana* (strain DPK-02-d), *Metarhizium anisopliae* (strain DPK-06-d) and *Isaria farinosa* (strain DPK-06-f-11) were tested on pine defoliator Bordered White moth (Pine Looper), *Bupalus piniaria* (L.) 3rd and 4th instars larvae. Although all three fungal strains were capable to kill *B. piniaria* larvae, the differences among strains were evident. *B. bassiana* (strain DPK-02-d) was the most efficient agent for a *B. piniaria* larvae control following by *M. anisopliae* (DPK-06-d) and *I. farinosa* (DPK-06-f-11) strains.

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PUŠŲ SPYGLIŲ KENKĖJAS *BUPALUS PINIARIA* (L.) (LEPIDOPTERA, GEOMETRIDAE) IR SU JUO SUSIJĘ ENTOMOPATOGENINIAI MIKROMICETAI. 2. MIKROMICETŲ *BEAUVERIA BASSIANA*, *METARHIZIUM ANISOPLIAE* IR *ISARIA FARINOSA* PATOGENIŠKUMAS

S a n t r a u k a

Lietuvoje išskirtų mikromicetų *Beauveria bassiana*, *Metarhizium anisopliae* ir *Isaria farinosa* kapienų (konidijų suspensijų koncentracija – 1×10^8 konidijų mL^{-1}) poveikis 3 ir 4-o ūgio pušinio sprindžio (*Bupalus piniaria*) vikšrams buvo tirtas laboratorinėmis sąlygomis. Visi trys mikromicetai slopino vikšrų gyvybingumą, tačiau *B. bassiana* virulentiškumas buvo didžiausias: per 12 dienų po purškimo mirtingumas pasiekė 100 %. *M. anisopliae* virulentiškumas 100 % pasiekė per 18 dienų. Nedidelis *B. piniaria* vikšrų mirtingumas nustatytas naudojant *I. farinosa* konidijų suspensiją: netgi pakartojus purškimą, mirtingumas tesiekė $56,67 \pm 6,6$ %. *B. bassiana* gali būti efektyvus entomopatogenas *B. piniaria* vikšrams.

Raktažodžiai: biologinė kontrolė, pušinis sprindis, miško kenkėjai, vabzdžių patogenai, endopatoziniai grybai