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

1. Fungi isolation and testing on larvae

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Pine defoliator bordered white moth (pine looper), *Bupalus piniaria* L. (Lepidoptera: Geometridae) larvae were reared under laboratory conditions and were regularly supplied with pine twigs collected in nature for feeding. Cadavers of naturally infected 2nd and 3rd stage larvae were collected and analysed. Thirty-six fungal isolates belonging to 15 species and 10 genera were obtained, cultivated and identified. Among them two species prevailed: *Lecanicillium psalliotae* (syn. *Verticillium psalliotae*) and *Fusarium solani*, comprising respectively 34.6 and 24.3% of the total number of fungi isolates. Conidial suspensions of the two fungi species at concentrations 10^5 to 10^8 conidia/ml were tested. Only the highest concentration of *F. solani* induced the mortality of 4th instar larvae, although the virulence was low: accumulative mortality ranged from 29.6 to 30.7% after 10 days of spraying. In the control group, the mortality was 15.8% after the same period. The high percentage of cadavers containing one of the two fungal species and the low mortality recorded in the test could be due to the very different sensitivity to the pathogens in different stage larvae. This is the first report on the isolation and identification of fungi developing on *B. piniaria*.

Key words: pine looper, forest pests, insect pathogens, mortality, microscopic fungi

INTRODUCTION

Bordered white (or pine looper) moth, *Bupalus piniaria* L. (Lepidoptera: Geometridae) occurs in coniferous woodlands throughout Europe and northern Asia and is mainly associated with pine trees, especially Scots pine (*Pinus sylvestris* L.). The moth is among the most important pests in pine stands. Caterpillars of the species feed on pine needles and are able to defoliate even mature trees. Damaged trees are more easily attacked by stem-attacking and other pests (Cedervind et al., 2003), the damage causes timber loss (Straw, 1996, 2002) and even tree death in case of defoliation during a few years in a row (Långström et al., 2004). During bordered white moth outbreaks, thousands of hectares of pine stands can be defoliated, e. g., 7000 ha in 1996 in Sweden (Cedervind et al., 2003).

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For population dynamics of *B. piniaria* as well as of any other insect species, both biotic and abiotic factors are very important. Among the former factors, the effect of parasitoids is rather well known (e. g., Ford, Shaw, 1991); however, as far as we know, data on the effect of entomopatogenous fungi are absent. There were no reports on fungi species causing death of bordered white moth larvae or on the scale of their impact on nature. It is difficult to investigate the interaction between fungi and *B. piniaria* under natural conditions because caterpillars dwell high in a canopy of pine trees, thus making it difficult to collect and analyse both healthy larvae and cadavers. Besides, pines as well as other conifers, release into the environment relatively large amounts of phytoncides, i. e. chemical substances suppressing activity of microorganisms, including bacteria and fungi. Released for plant protection, phytoncides could provide some protection for phytophagous insects as well. On the other hand, some fungi in the course of

evolution could become adapted to the environment of a tree canopy rich in phytoncides.

The purpose of the present paper was to isolate and identify fungi damaging *B. piniaria* larvae while being reared under laboratory conditions on pine needles as well as to evaluate the pathogenicity of fungal strains of two most often registered species.

MATERIALS AND METHODS

Insects. Pupae of bordered white moth, *Bupalus piniaria* L. (Lepidoptera: Geometridae) were collected under litter of a pine stand in southern Lithuania (Druskininkai district) in late autumn 2007. For overwintering, the pupae were kept in conditions close to natural. In spring, after adult emergence, they were mated in cages and supplied with pine (*Pinus sylvestris* L.) twigs for egg laying. Hatched larvae were supplied with fresh needles on 10–15 cm long pine twigs for feeding. The twigs were collected in nature from different trees and replaced regularly to ensure suitable feeding conditions. Larvae were reared under laboratory conditions at 19–22 °C. Cadavers of the second and third stage larvae were collected regularly.

Isolation and identification of fungi. Fungi grown in the cadavers of dead *B. piniaria* larvae were isolated and identified. After removing from the rearing cage, the surface of each cadaver was sterilized in 5% sodium hydrochloride and 75% ethanol solution and rinsed in plenty of sterile distilled water. The cadavers were left to dry for 48 h. After drying they were incubated in clean desiccators (each sample in a separate Petri dish) at room temperature in humid conditions. Sporulating cadavers were regarded as being infected by a fungus, while non-sporulating cadavers were regarded as not infected. The sporulating fungi from cadavers were isolated and transferred on Sabourand Dextrose Agar (SDA, Liofilchem, Italy) with the aim to cultivate them for species identification, on three growth media: potato dextrose agar (PDA, Liofilchem, Italy), malt extracts agar (2% MEA, Liofilchem, Italy) and Czapek's agar (CA, Liofilchem, Italy). To confirm the genus *Fusarium* Link the mycelium of one morphotype was transferred from the selective media on a carnation leaf agar (Fisher et al., 1982) and then repeatedly transferred on PDA medium to facilitate fungus identification, using the taxonomy recommended by Nelson et al. (1983). Fungi were grown at 24 °C in the dark. Other fungi were identified according to the keys of E. Kiffer et al. (1997) and K. Domsch et al. (2007).

Two fungal strains, *F. solani* (strain DPK-08-f-5) and *L. psalliotae* (strain DPK-08-v-6), were used for the pathogeneicity bioassay against *B. piniaria* larvae. Fungi were cultivated at 25 °C, photoperiod 12 h of light and darkness (12 h L : D) for 10 days. After incubation, a sterile spatula was used to harvest conidia from the fungal culture. The fungal culture grown in a plate was flooded with sterile 0.05% Tween 80 (Sigma USA) water solution. The conidial suspensions were passed through two layers of a cheese cloth to remove any large particles and hyphae. The harvested conidia were transferred into sterile bottles. Then a fungal conidia suspension in sterile 0.05% Tween 80 water solution was prepared, and the total number of the conidia in the suspensions was determined with a heamocytometer. The concentration of viable conidia in one ml of conidial suspension prepared for spraing was calculated from the total number, and the germination percentage was determined on a PDA medium. The viability of the fungal conidia was determined after 7 and 14 days of the cultivation in *F. solani* and *L. psalliotae*, respectively. The germination tests were performed placing a layer of the potato dextrose agar (PDA, Liofilchem, Italy) medium on microscope slides and adding drops of the conidial suspension. Prepared slides were incubated in moist chambers. The percentage of germinated conidia was determined after 16–24 hours of incubation in moist chambers in the dark. Prepared stock suspensions were diluted to 1.4×10^8 conidia/ml. Conidial suspensions were sprayed on pine twigs with a glass sprayer.

Fungal activity test. The forth instars of *B. piniaria* larvae were transferred and launched on pine twigs and moved into glass cages (750 ml in volume). The daily mortality of *B. piniaria* larvae was recorded both in control (cages containing larvae on pine twigs sprayed with distilled water) and experimental cages (containing larvae on pine twigs sprayed with a conidial suspension of a needed species and concentration). Each treatment was in triplicate. The mortality of larvae was recorded during a three-week period at 20 ± 1 °C, at a relative humidity >65% and a 12 : 12 light : dark photoperiod. Each larval cadaver was transferred into Petri dish supplied with moistened filter paper and was incubated at 24 °C in the dark. Symptoms of deceased larvae suggesting that they died due to fungal infection confirmed the accuracy of the experiment. When larvae were covered by fungus mycelium, re-identification was made according to the micro-morphology using keys as indicated above. Slide cultures were used for microscopic examination of intact fungal reproductive structures. To prepare the slides, melted MEA medium (70 °C) was placed on a sterile microscopic slide in the amount sufficient to cover half of the length of the slide surface and three quarters of its width with a thin, flat layer of agar. When the agar solidified, the fungus was inoculated on the surface by streaking spores in two parallel rows extending the length of the agar layer. The slide cultures were incubated at 24 °C in a humid chamber and were allowed to grow (for 4–7 days) until the reproductive structures appeared.

Statistical analysis. To obtain statistically significant data in the fungal activity test, groups of 10 insects per test were used for spraying. Both control and each test were performed in three replicates. Standard error was estimated for every experimental point and marked in Fig. 1 as an error bar. Statistical significance in mortality was evaluated by the Wilcoxon matched pairs test (criterion Z).

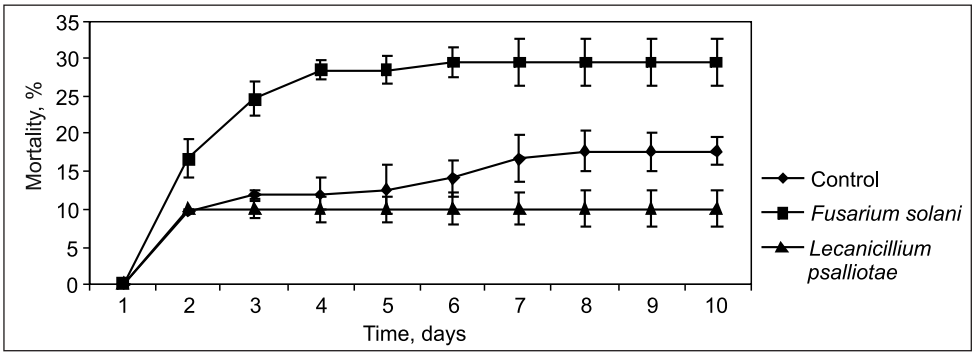

Fig. 1. Percentage mortality (mean percentage ± SD) of *B. piniaria* larvae treated with *F. solani* and *Lecanicillium psalliotae* conidia (concentration 1.4×10^8 ml^{-1} or water control without fungal conidia

RESULTS AND DISCUSSION

Fungi isolated from B. piniaria larval cadavers

In total, 56 cadavers were subjected to determining fungal infections. Presence of fungi was recorded in 86% of the cadavers.

Thirty-six fungal isolates belonging to 15 species and 10 genera were isolated, cultivated and identified (Table). As far as we know, there were no reports on the isolation and identification of fungi developing on *B. piniaria*, thus the obtained data could be compared with the results recorded on other species only.

Lecanicillium psalliotae (Treschew) Zare, W. Gams (syn. *Verticillium psalliotae*) was the most common species comprising 34.61% of the total number of the fungus isolates. According to the description of R. Zare, W. Gams (2001), our isolate is similar to the fungus isolated from scale insects of palm in Spain, which was provisionally assigned to *L. psalliotae*. As to the nomenclature of the species based on the recent taxonomic publications, the correct name of the species is either *Lecanicillium fungicola* var. *fungicola* (synonyms: *L. psalliotae*, *L. malthousei*) after G. H. Sung et al. (2007) or *L. psalliotae*, according to the monograph of K. Domsch et al. (2007). We prefer to follow the latter taxonomist who left the name of the species valid. Besides, it should be noted that *L. psalliotae* is known as a nematophagous fungus (Gan et al., 2007), a well known biocontrol agent of ticks (Pirali-Kheirabad et al., 2007), and the general pathogen of various soil insects (Kurihara et al., 2006). The nematophagous fungus *L. psalliotae* (syn. *V. psalliotae* used in the cited paper) is a well-known biocontrol agent used in China (Gan et al., 2007).

Table. **Fungi isolated from cadavers of Bupalus piniarius larvae**

Class	Order	Family	Genus	Species	Percentage
Dothideomycetes	Capnodiales	Davidiellaceae	*Cladosporium* Link	*C. brevicompactum* Rebrik. & Sizova	1.42
Sordariomycetes	Hypocreales	Cordycipitaceae	*Beauveria* Vuill.	*B. bassiana* (Bals.-Criv.) Vuill.	0.27
			Paecilomyces Bainier	*P. farinosus* (Holmsk.) A. H. S. Brown & G. Smith.	2.14
			Lecanicillium W. Gams & Zare	*L. lecanii* (Zimm.) Zare & W. Gams	0.82
				L. psalliotae (Treschew) Zare & W. Gams	34.61
				L. tenuipes (Petch) Zare & W. Gams	0.20
		Nectriaceae	*Fusarium* Link	*F. solani* (Mart.) Sacc.	24.33
				F. subglutinans (Wollenw. & Reinking)	
				P. E. Nelson, Toussoun & Marasas	0.61
				T. roseum (Pers.) Link	6.21
Eurotiomycete	Eurotiales	Incertae sedis	*Trichothecium* Link	*A. flavus* Link	3.51
		Trichocomaceae	*Aspergillus* P. Micheli ex Link	*P. frequentans* Westling	2.93
			Penicillium Link	*P. solitum var. crustosum* (Thom)	
				Bridge, D. Hawskw., Kozak., Onions, R. R. M. Peterson & Sackin.	6.3
Zygomycetes	Entomophthorales	Entomophthoraceae	*Massospora* Peck.	*M. cleoni* Wize	1.27
	Mucorales	Mucoraceae	*Mucor* Fresen.	*M. hiemalis* Wehmer	8.83
				M. ramosissimus Samouts.	6.48

As *L. psalliotae* was isolated from *B. piniaria* larval cadavers most often during insect rearing, the culture of the fungus was prepared with the aim to test its entomopathogenic effect and to reveal the expected high mortality effect. Conidia at a concentration of 1.4×10^8 ml^{-1} were sprayed on pine twigs containing the fourth instar larvae of *B. piniaria*. No statistically significant mortality was recorded during 10 days after the application; moreover, a trend towards mortality decrease compared to the control was noted (Fig. 1).

A discrepancy between almost non- virulent *L. psalliotae* recorded in the test and the high percentage of cadavers damaged by the fungus recorded during rearing should be stated. It should be emphasised that the cadavers were collected as 2nd and 3rd stage larvae, while the test was performed on the 4th stage larvae. Thus, in our opinion, the discrepancy could be explained by a much higher resistance towards fungi in the last stage larvae.

Fusarium solani (Mart.) Sacc. was the second species among the fungi most often isolated from *B. piniaria* cadavers, and comprised 24.33% of the total number of infected cadavers. *F. solani* is known as an important plant pathogen (Nelson et al., 1983). However, *F. solani* was also recorded as associated with insects, e. g., beetles (*Coleoptera*), flies (*Diptera*) and some moths (Rojas et al., 1999; Majumdar et al., 2007; Big-Da, Xin-Zhogn, 2007). It is primarily a soil-borne fungus naturally occurring in a wide range of agricultural, grassland and forest nursery soils (Summerell et al., 1993; James et al., 1996).

As *F. solani* was the second species among the most often isolated fungi, a culture of the fungus isolate was prepared to evaluate its entomopathogenic effect. The fourth instar *B. piniaria* larvae on pine twigs were sprayed with a suspension of conidia at a concentration of 1.4×10^8 ml^{-1}. The mortality of larvae was significant starting from the 4th day after spraying. However, the effect was low: mortality reached 29.6 to 30.7% 10 days after sprayng, and mortality in the control was 14.3 to 17.2% during the same period.

Some discrepancy in the low virulence of *L. psalliotae* recorded in the test and the high percentage of cadavers damaged by the fungus collected in rearing cages could be explained, to some extent, by a higher resistance towards fungi in larvae of the last stage.

Mucor hiemalis Wehmer was isolated from 8.83% of the cadavers. *M. hiemalis* is known as a secondary colonizer of insect cadavers (Samson et al., 1988; Tanada, Kaya, 1993). However, its toxicity to adult flies (Diptera: Tephritidae) of two species (*Bactrocera oleae* and *Ceratitis capitata*) was demonstrated (Konstantopoulou et al., 2006). Feeding and contact bioassays revealed a strong toxicity with quickly developed symptoms of toxicity, lethargy (within 1–2 h post-treatment), and mortality (82–97% after 24 h). *Mucor hiemalis* f. *hiemalis* was also isolated from spider samples (Netwing, Prillinger, 1990).

Penicillium solitum var. ***crustosum*** (Thom) Bridge, D. Hawskw., Kozak., Onions, R. R. M. Peterson, Sackin. (syn. *Penicillium crustosum* Thom) was isolated from 6.30% of cadavers. The insecticidal property of the fungus was demonstrated by M. X. C. González et al. (2003).

Mucor ramosissimus Samutsevitsch was isolated from 6.48% of the cadavers. The species was attributed to saprobic fungi and was known as related to some bird feather diseases (Qesada et al., 2007). The fungus was also isolated from *Varroa* mites (Hrabak, 2005), but no reports on its relation to insects have been published so far.

Trichothecium roseum (Pers.) Link was isolated from 6.21% of cadavers. The fungus is a well known endophytic plant pathogen; it occurs also in soil and stored grain (Domsch et al., 2007). It is nontoxic and even suitable for food in specialised stored grain pests beetles, e. g., *Cryptolestes ferrugineus* and *Oryzaephilus mercator* (Coleoptera) (Sinha, 1965). M. F. Madelin (1996) has reported that *T. roseum* is not a virulent pathogen for many insects. On the other hand, the toxin from *T. roseum* was identified (named as Roseotoxin B) and patented as a means for Lepidopteran pest control (Dowd, Cole, 1990). This fungus appeared to be a secondary pathogen or saprobe rather than a primary pathogen for *B. piniaria* larvae.

Penicillium frequentans Westling (syn. *Penicillium glabrum*) was isolated from 2.93% of cadavers. *P. frequentans* was known as a common soil fungus with a high percentage in soil fungus communities (Domsch et al., 1993).

Aspergillus flavus Link. is mainly saprophytic but may infect a wide range of insect species (Tanada, Kaya, 1993). In *B. piniaria* larvae, infections were determined in 3.51% of cases. The fungus is attributed to opportunistic fungi capable to infect a wide variety of hosts, including plants, insects and mammals, although with a low virulence (Scully, Bidichka, 2006). The results show a broad geographical occurrence of *A. flavus* on insects feeding on developing corn (Tanada, Kaya, 1993). *A. flavus* was isolated from *Lygus hesperus* (lygus bug) (Stephenson, 1974), its toxin, kojic acid, is highly insecticidal at high concentrations to the house fly maggot (Beard, Walton, 1969), other toxins, such as B_1, B_2, G_1 and G_2, are toxic when fed to larvae of several insect species (e. g., *Heliothis virescens*, *Spodoptera frugiperda*, *S. litoralis*, *Ostrinia nubilalis*, *Sitophilus zeamais*, and *Drosophila melanogaster*) (McMillian et al., 1981).

Paecilomyces farinosus (Holmsk.) A. H. S. Brown, G. Smith Fr. (syn. *Isaria farinosa*) was isolated from 2.14% of *B. piniaria* cadavers. The species is distributed worldwide with a relatively wide host range. Over the past 40 years, numerous scientific papers on *P. farinosus* have been published. G. Zimmermann (2008) presented a review on the biology, ecology and usage of *P. farinosus* as a biocontrol agent.

Lecanicillium lecanii (Zimm.) Zare, W. Gams (syn. *Verticillium lecanii*) was isolated from 0.82% of *B. piniaria* cadavers. This fungus is one of the most important entomopathogenic hyphomycetes and was isolated from scale insects and aphids, trips, flies, species from Hymenoptera and Lepidoptera orders, as well as from mites. The fungus has been commercialized as a biopesticide against aphids (Domsch et al.,

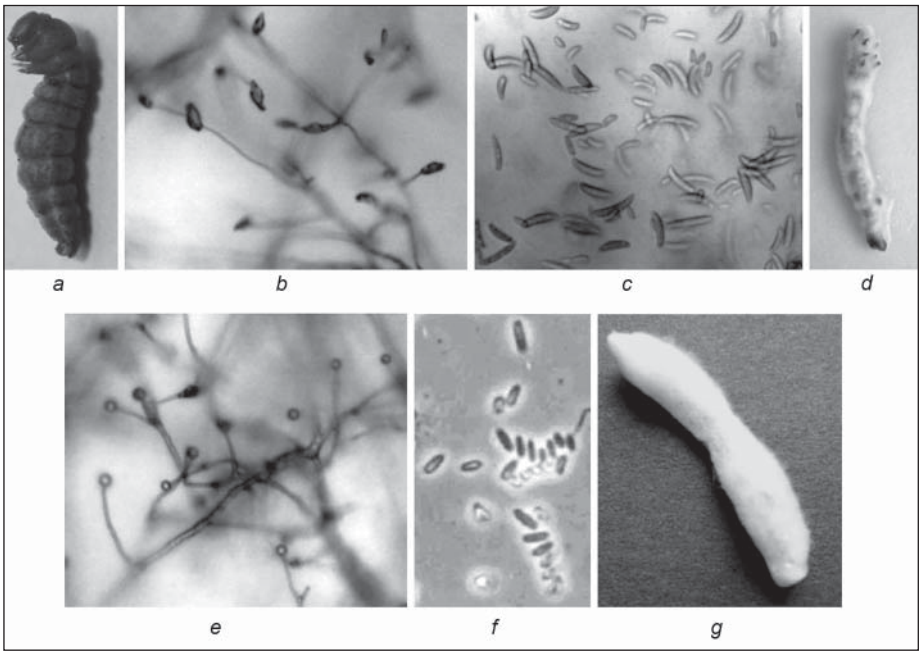

Fig. 2. Mature larvae (*a*) of *B. piniaria* and the morphology of treated fungi grown on larvae cadavers after 3–5 days. *Fusarium solani*: conidiophores with conidial heads (*b*), conidia (*c*) and mycelium grown on the cadaver (*d*). *Lecanicillium psalliotae*: conidiophores with conidial heads (*e*), conidia (*f*) and mycelium grown on the cadaver (*g*)

1993) and for whitefly control (Milner, 1997). The host range of the species is wide and includes some other homopteran insects as well as a range of other arthropod groups. The potential of *L. lecanii* for managing insects, nematodes and plant diseases is well documented (Goettel et al., 2008). Like other entomopathogenic fungi, *L. lecanii* produces chitinases able to degrade effectively the cuticle of various insects, and this aspect highlights the biocontrol potential of this fungus to insect pests (Harper, Huang, 1986).

Fusarium subglutinans (Wollenw. & Reinking) P. E. Nelson, Toussoun & Marasas. In *B. piniaria* larvae, infections were determined in 0.61% of cases. The fungus is a well known plant pathogen. Its toxicity to lepidopterans was demonstrated at a cell level only (Logrieco et al., 1996).

Massospora cloeni Wize was isolated from 1.2% of *B. piniaria* cadavers. Within the genus *Massospora* species, entomopathogenic fungi for insects were revealed (e. g., Duke et al., 2002); however, data on *M. cloeni* species remains unknown.

Beauveria bassiana (Bals.-Criv.) Vuill. was isolated from 6.21% of cadavers. The species occurs worldwide, and its main natural hosts are usually insects from the orders *Lepidoptera*, *Coleoptera*, *Hemiptera*, *Diptera*, *Hymenoptera* and *Homoptera* (Kanga et al., 2004)

Cladosporium brevicompactum Rebrik & Sizova belong to common air-born fungi found also as endophytic and soil fungi (Domsch et al., 2007). In *B. piniaria* larvae, infections caused by this fungus were revealed in 1.42% of all entomopathogenic cases. Some authors consider *Cladosporium* spp. as a new promising biological control agent against homopterous and hemipterous insects (Abdel-Baky, 2000; Tanada, Kaya, 1993). Natural infestation of whiteflies and aphids by *C. brevicompactum* was registered within the range from 16.4 to 45.27% (Abdel-Baky, 2000).

Lecanicillium tenuipes (Petch) Zare & W. Gams (syn. *Verticillium tenuipes*) was isolated from 0.82% of *B. piniaria* cadavers. The fungus is found in soil and is classified as a keratinophylic fungus (Parihar, Kushwaha, 2000). It may cause some plant diseases (Singh et al., 2009). *L. tenuipes* exhibits a pronounced chitinolytic activity (Leinhos, 1992), therefore it can be associated with soil insects and / or nematodes.

Fungal activity test

Among the fungi species most frequently found in cadavers of *B. piniaria*, two species – *F. solani* and *L. psalliotae* – prevailed. Thus, these two species were chosen to evaluate their entomopathogenic activity.

Two strains, DPK-08-f-5 of *F. solani* and DPK-08-v-6, of *L. psalliotae* were tested. A different impact on insect mortality of these two strains was revealed. In the treatment with *F. solani*, larvae of *B. piniaria* were sprayed with the fungus conidial suspension at a concentration 1.4×10^5 to 1.4×10^8 ml^{-1}. Only conidial suspensions at concentrations 1.4×10^8 ml^{-1} induced a statistically significant mortality of the larvae (Fig. 1), however, with a low virulence: the accumulative mortality percentage reached only 29.6–30.7% after 10 days after spraying. The mortality of larvae spayed with *F. solani* was approximately only twice higher compared to the control (14.2–15.6% in the control). Some mortality was induced by the concentrations 1.4×10^7 ml^{-1} as well, but lower concentrations didn't induce mortality as compared to control ($p > 0.05$).

The conidial suspension of *L. psalliotae* used at the same concentration as that of *F. solani* (1.4×10^8 ml^{-1}) was not effective against *B. piniaria* larvae in a laboratory test (Fig. 1). Mortality percentage in cages with *L. psalliotae* was even lower than in the control. As the fungus is known to be active against nematodes (Sung et al., 2007, Gan et al., 2007),

and assuming that some larvae could be contaminated with these parasites, a slight positive effect of the fungus could be attributed to their suppression.

To confirm the mortality effect caused exactly by the fungal species used for spraying, larval cadavers were removed from the cages and incubated in a wet chamber. On larvae damaged by *F. solani*, mycelium appeared after one day (Fig. 2, *d*) and conidia were formed after two days of incubation (Fig. 2, *b* and *c*). This fungal species was detected in all dead larvae transferred from cages with *F. solani* tests. Such results suggest the susceptibility of *B. piniarius* larvae to the fungus *F. solani* isolated from the naturally infected cadavers. Among the fifteen fungal species isolated and tested, *F. solani* was the most common (Table). A laboratory test confirmed the virulence of the fungus, although not high.

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PUŠŲ KENKĖJAS *BUPALUS PINIARIA* L. (LEPIDOPTERA, GEOMETRIDAE) IR SU JUO SUSIJĘ ENTOMOPATOGENINIAI MIKROMICETAI

1. GRYBŲ IŠSKYRIMAS IR POVEIKIO VIKŠRAMS ĮVERTINIMAS

S a n t r a u k a

Pušis galinčio visiškai defoliuoti kenkėjo – pušinio sprindžiaus (*Bupalus piniaria*) vikšrai, išsiritę iš kiaušinėlių, buvo auginami laboratorijos sąlygomis ir maitinami spygliais, pateikiamais su pušų šakelėmis, surinktomis gamtoje. Iš 2 ir 3 ūgio žuvusių vikšrų buvo išskirti, užauginti ir identifikuoti 36 mikromicetai, priklausantys 15 rūšių, 10 genčių. Tarp jų vyravo 2 rūšys: *Lecanicillium psalliotae* (sin. *Verticillium psalliotae*) ir *Fusarium solani*, kurios sudarė atitinkamai 34,6 ir 24,3 % visų išskirtųjų mikromicetų. Tirtos šių grybų konidijų suspensijos (koncentracijos nuo 10^5 iki 1, 4 10^8 konidijų/ml) poveikis 4 ūgio vikšrams. Tik *F. solani* slopino vikšrų gyvybingumą, tačiau virulentiškumas buvo nedidelis: per 10 dienų po purškimo mirtingumas tepasiekė 29,6–30,7 %; kontrolėje mirtingumas per tą patį laikotarpį – 17,5 %. Nedidelis *B. piniaria* mirtingumas aiškintinas paskutinio ūgio vikšrų atsparumu patogenui. Duomenų apie mikromicetų išskyrimą ir identifikavimą iš *B. piniaria* vikšrų iki šiol nebuvo.

Raktažodžiai: pušinis sprindžius, miško kenkėjai, vabzdžių patogenai, mirtingumas, entomopatogeniniai grybai