ACTA ZOOLOGICA BULGARICA
Acta zool. bulg., 65 (2), 2013: 179-186

Fungal Pathogens on Some Lepidopteran Forest Pests in Bulgaria

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Abstract: Fungal entomopathogens found in natural populations of 7 lepidopteran forest pests were isolated and identified. *Beauveria bassiana* was established as a dominating species followed by *Fusarium* sp. and *Aspergillus* sp. The pine processionary moth *Thaumetopoea pityocampa* was the most affected by *B. bassiana* – 66.7% of the analysed dead larvae of the pest. The occurrence of *B. bassiana* in *Malacosoma neustria*, *Tortrix viridana* and *Melitaea didyma* is the first record in Bulgaria.
Relative susceptibility of first-second instar *Lymantria dispar* larvae was estimated to three *B. bassiana* and one *Metarhizium anisopliae* isolate and susceptibility of third instars – to seven *B. bassiana* and one *M. anisopliae* isolate. The bioassays showed that *L. dispar* larvae were tolerant to mycoses caused by the tested *B. bassiana* isolates and *M. anisopliae*. *M. anisopliae* isolate caused mycosis to first-second instar larvae with the highest lethal effect 56.67% ± 4.84 and *B. bassiana* isolates 561Bb and 575Bb to third instar larvae – 47.78% ± 4.06 and 34.44% ± 3.28, respectively, in 17 days period.

Key words: lepidopteran forest pests, entomopathogenic fungi, *Lymantria dispar*, *Beauveria bassiana*, *Metarhizium anisopliae*

Introduction

Lepidopteran species such as pine processionary moth *Thaumetopoea pityocampa* (Den. & Schiff.), the gypsy moth *Lymantria dispar* (L.), green oak leefroller *Tortrix viridana* L., *Malacosoma neustria* (L.), *Euproctis chrysorrhoea* (L.) and Geometridae species *Operophtera brumata* (L.) and *Erannis defoliaria* (Cl.) are among the most harmful pests in coniferous and deciduous forests registered in Bulgaria (Mirchev, Tsankov 2000). Their mass outbreaks could cause serious damages even to defoliate deciduous trees.

The existent IPM of lepidopteran forest pests include aerial spray treatments using several preparations of *Bacillus thuringiensis* var. *kurstaki* or chemical pesticides like Dimilin. Efforts to improve the existent environment friendly strategies were the reason to study fungal pathogens – their biodiversity (Papierok *et al*. 1984, Solter *et al*. 2000, 2002, Jankevica 2004, Mirchev 2004, Pilarska *et al*. 2001, 2006, 2010; Polovinko *et al*. 2010; Sevim *et al*. 2010), pathogenicity (Er *et al*. 2007; Polovinko *et al*. 2010; Sevim *et al*. 2010) and to conduct introduction of the entomophthorous fungus *Entomophaga maimaiga* Humber, Shimazu & Soper (Pilarska *et al*. 2000, 2007, 2010).

The aim of the study was to establish naturally occurring fungal pathogens on some lepidopteran forest pests in Bulgaria and to estimate the relative

susceptibility of first-second and third instar *L. dispar* larvae to 7 isolates of *Beauveria bassiana* (Bals. – Criv.) Vuillemin and one isolate of *Metarhizium anisopliae* (Metsch.) Sorokin.

Materials and Methods

Natural occurrence

The occurrence of fungal entomopathogens in natural populations of lepidopteran forest pests was studied in 2 year period from April 2009 to June 2011. A total of 563 individuals of insects (larvae, pupae, adults) belonging to 7 lepidopteran species were collected from 12 forest stands from different regions in Bulgaria (Table 1).

The found dead insects were collected in individual containers for laboratory analyses. The collected alive larvae were reared on natural diet (leaves of apple, plum or oak trees) in the laboratory till their death or imagination. All dead insects were analysed microscopically. Parts of cadavers of insects with symptoms of mycosis after surface sterilization were placed in a humid chamber at temperature of 25 ± 1 °C for sporulation of fungal pathogens. Conidia from sporulated fungi were transfered to SDAY (Sabouraud dextrose agar with yeast extract) in glass Petri dishes and cultured for 15 days at 25 ± 2 °C at a photoperiod of L12:D12. The obtained pure cultures of the isolates after subculturing on slopes of the same media in tubes were kept in a refrigerator at temperature of 4 ± 2 °C. Conidia from pure cultures of isolates and small parts of cadavers were used to prepare smears stained by methylen-blue as well as slide preparations with lactophenol and aniline blue closed by nail vanish (after Humber 1997). Conidia and conidiogenous cells were observed using a transmission interference microscope BX60 DIC Olympus equipped with digital camera and Cell B image capture software with calibrated Carl Zeiss micrometer. The fungal pathogens were identified according to their macro- and micro-morphological characteristics according to Samson *et al*. (1988) and Humber (1997).

Bioassays with fungal isolates and larvae of *L. dispar*

Seven isolates of *B. bassiana* obtained from different lepidopteran and coleopteran forest insects and one isolate of *M. anisopliae* from a dead adult of the grey corn weevil were used in bioassays (Table 2). They were cultured for 15 days on slopes of SDAY in tubes under the conditions mentioned above and obtained conidia were washed down with sterilized water containing 0.05% Tween 80. The concentrations of conidia were determined by enumeration of conidia in Bürker chamber after serial dilution of aqueous suspensions. Suspensions applied in bioassays were prepared at a concentration of $1x10^8$ conidia/ml.

First-second and third instar *L. dispar* larvae used to estimate the relative susceptibility to fungal isolates were from a laboratory population reared on natural diet (plum-tree leaves) at temperature of 22 ± 1 °C and 70 ± 1% RH. Insects were obtained after hatching of gypsy moth eggs provided by the USDA-APHIS-PPQ Laboratory, Buzzards Bay, MA, USA. The insects were treated by a surface contact with 1 ml of conidial suspensions ($1x10^8$ conidia/ml) placed on filter paper discs (90 mm in diameter) in glass Petri dishes (Draganova, Staneva 1990). The larvae in control variants were treated with 1 ml of water containing 0.05% Tween 80 instead of conidial suspension. The filter paper disks were removed 24 h after the treatments and insects were fed on plum-tree leaves. The experiments were conducted under laboratory conditions at temperature of 22 ± 1 °C and 70 ± 1% RH in three replicates with 30 first-second or third instar *L. dispar* larvae per a replicate. Insect mortality was checked daily for 17 days after contamination. Dead insects in all treatments were removed daily and were placed on moist filter paper in new Petri dishes for a fungal pathogen exhibition. The results of the bioassays were evaluated as percentages of cumulative daily mortality due to mycoses, corrected with mortality in the control treatment following the Schneider-Orelli's formula (Püntener 1981). Differences among lethal effects of mycoses to *L. dispar* larvae in treatments with conidial suspensions of fungal isolates were estimated by t-test for independent samples applying Descriptive statistics. Values of $p < 0.05$ were considered significant. Statistical analyses were performed using the software STATISTICA^R version 6.0 of Stat Soft Inc.

Results and Discussion

Natural occurrence

The analyses of 145 dead individuals from 7 lepidopteran species showed that 89 of them were with symptoms of mycoses (Table 3). Fungal patho-

Table 1. Lepidopteran forest pests.

Species, family	Locality
Thaumetopoea pityocampa (Den. et Schiff.), Notodontidae	Banya (Karlovo district)
Lymantria dispar L., Lymantriidae	Pobit kamak (Sredna Gora Mountain) Konush (Eastern Rhodopes) Perperek (Eastern Rhodopes) Gnyazdovo (Eastern Rhodopes) Krumovgrad (Eastern Rhodopes) Nanovitza (Eastern Rhodopes) Zvezdel (Eastern Rhodopes) Kamenetz (Eastern Rhodopes)
Aporia crataegi L., Pieridae	Kamenetz (Eastern Rhodopes)
Malacosoma neustria L., Lasiocampidae	Gnyazdovo (Eastern Rhodopes) Kamenetz (Eastern Rhodopes)
Erannis defoliaria Clerck, Geometridae	Gnyazdovo (Eastern Rhodopes) Perperek (Eastern Rhodopes)
Melitaea didyma Esper, Nymphalidae	Krumovgrad (Eastern Rhodopes)
Tortrix viridana L., Tortricidae	Plakovo (Central Balkan Range) Pobit kamak (Sredna Gora Mountain) Kardjali (Eastern Rhodopes) Perperek (Eastern Rhodopes) Gnyazdovo (Eastern Rhodopes) Komuniga (Eastern Rhodopes) Krumovgrad (Eastern Rhodopes) Zvezdel (Eastern Rhodopes)

Table 2. Origin of the isolates of entomopathogenic fungi applied in bioassay with *Lymantria dispar* larvae (L_{1-2} and L_3)

Isolate, entomopathogenic fungus	Origin (initial insect host)		Year of isolation
	Species	Stage	
560Bb, *B. bassiana*	*Thaumetopoea pityocampa* (Den. et Schiff.) (Lepidoptera: Notodontidae)	larva	2009
561Bb, *B. bassiana*	*Stenomax aeneus* (Scop.) (Coleoptera: Tenebrionidae)	imago	2009
575Bb, *B. bassiana*	*Lymantria dispar* L. (Lepidoptera: Lymantriidae)	larva	2009
588Bb, *B. bassiana*	*Tortrix viridana* L. (Lepidoptera: Tortricidae)	larva	2010
593Bb, *B. bassiana*	*Operophtera brumata* L. (Lepidoptera: Geometridae)	larva	2010
596Bb, *B. bassiana*	*Thaumetopoea pityocampa* (Den. et Schiff.) (Lepidoptera: Notodontidae)	larva	2010
601Bb, *B. bassiana*	*Thaumetopoea pityocampa* (Den. et Schiff.) (Lepidoptera: Notodontidae)	larva	2010
619Ma, *M. anisopliae*	*Tanymecus dilaticollis* Gyll. (Coleoptera: Curculionidae)	imago	2010

gens were identified as *Beauveria bassiana* (Bals. – Criv.) Vuillemin (Sordariomycetes: Hypocreales, Cordycipitaceae), *Fusarium* sp. (Sordariomycetes, Hypocreales, Nectriaceae) and *Aspergillus* sp. (Eurotiomycetes, Eurotiales, Trichocomaceae). Fifty seven *B. bassiana* isolates were obtained in pure cultures on SDAY.

The established results revealed that *B. bassi-*

Table 3. Pathogens established in natural populations of lepidopteran forest pests

Lepidopteran host			Pathogen (number of infected individuals)
Species, family	**Total number of investigated individuals**	**Number of dead individuals**	
Thaumetopoea pityocampa (Den. et Schiff.), Notodontidae	39 L 31 P	33 L 11 P	*B. bassiana* (22 L) *Fusarium* sp. (5 L), *Aspergillus* sp. (6 L, 7 P) Other (4 P)
Lymantria dispar (L.), Lymantriidae	59 L 8 P 2 i	33 L 3 P 1 i	*B. bassiana* (4 L, 1 P) Aspergillus sp. (2 L, 2 P) Other (27 L, 1 i)
Malacosoma neustria (L.), Lasiocampidae	131L	29L	*B. bassiana* (9 L) *Fusarium sp.* (2 L) Other (18 L)
Tortrix viridana L., Tortricidae	238 L 34 P 3 i	16 L 5 P 2 i	*B. bassiana* (10 L, 2 P, 2 i) *Fusarium* sp. (1 L) *Aspergillus* sp. (5 L) Other (3 P)
Erannis defoliaria (Cl.), Geometridae	16 L	10 L	*B. bassiana* (6 L) *Aspergillus niger* (2 L) Other (2 L)
Aporia crataegi (L.), Pieridae	1 L	1 L	Other (1 L)
Melitaea didyma (Esp.), Nymphalidae	1 i	1 i	*B. bassiana* (1 i)

*L – larva, P – pupa, i – imago
**Other – reason of death different from mycosis

ana was the fungal pathogen that occurred in natural populations of 6 from 7 species of the observed lepidopteran forest pests.

The larvae of the pine processionary moth were the most affected by the fungus – 66.7% of the analysed dead larvae. Twenty two *B. bassiana* isolates were obtained in pure cultures from them. The reason of such high extensity observed was that *B. bassiana* as soil-dwelling fungus can easily come into contact with the premature stages of *T. pityocampa* during their staying in the soil and infect them. Fungi are active pathogens which attack their hosts percutaneously. They produce extracellular enzymes lysing the host cuticula and penetrate through the arthropod integument with support of mechanical pressure of the growing tubes of the germinating conidia (St. Leger *et al.* 1986, Draganova 1988, Gupta *et al*. 1992; St. Leger 1995).

Our investigations established that *B. bassiana* occurred as a causal agent of mycoses in natural populations of *L. dispar* (7.2% of all collected and 13.5% of dead individuals), *M. neustria* (6.9% of all and 31% of dead individuals), *T. viridana* (5.1% of all and 60.9% of dead individuals), *E. defoliaria* (37.5% of all observed and 60% of dead individuals). Dead by mycoses were mainly larvae, rarely pupae. Few adults of some lepidopteran hosts (*T. viridana* and *M. didyma*) were found to be affected by the fungal pathogen as well (Table 3). This is the first record of *B. bassiana* in *M. neustria*, *T. viridana* and *M. didyma* in Bulgaria.

Panajotov *et al*. (1960) reported that the fungi *B. bassiana*, *B. glubulifera* Pic. and *Spicaria farinosa* Vuill. (now *Isaria farinosa* (Holmsk.) Fries) were found on *L. dispar* larvae in Bulgaria. Hajek *et al*. (1997) found out that *Paecilomyces farinosus* (Holmsk. ex Gray) Brown & Smith) (now *I. farinosa*) was the most often occurring hyphomycete species in natural populations of gypsy moth although in low infection level (4.6% – 12.2%) and rarely *B. bassiana.* According to Goettel *et al*. (1990) and Butt *et al*. (2001) *B. bassiana* is an entomopathogenic fungus with a large host range which includes about 700 arthropod species.

Polovinko *et al*. (2010) reported that *B. bassiana* was a dominating species comprising on average 68% of the total number of isolates from Lepidoptera and Coleoptera in West Siberia, Primorsky Krai, and Kyrgyzstan. During observation period the mortality rate of the insects died of *B. bassiana* was registered mostly on enzootic level although in some cases the local nidi of mass death of the hosts were noted. *Isaria farinosa* (Holmsk.) Fries was the entomopathogenic fungus found primarily on Lepidoptera – the Macroheterocera group. According to Polovinko *et al*. (2010) *Isaria farinosa* was also likely to have a significant epizootic potential. It was revealed that in some places in the region of Novosibirsk the mentioned fungus was present in soil in great amounts.

Fungal entomopathogens found to cause mycoses in natural populations of *T. pityocampa* were studied by Sevim *et al*. (2010). Based on their morphological and molecular characteristics four isolates were identified by the authors as *Beauveria bassiana* cf. Clade C, and one isolate was identified as *B. bassiana*.

The occurrence of *B. bassiana* in natural populations of *T. pityocampa* and *L. dispar* in Bulgaria and study of some properties of the obtained fungal isolates was reported by Draganova *et al*. (2011).

Another representative of genus *Beauveria* – *B. brongniartii* (Saccardo) Petch was announced by Jankevica (2004) to infect European tent caterpillar *M. neustria* in a study on the occurrence of entomopathogenic fungi and their host range.

The fungal species from genera *Fusarium* and *Aspergillus* found by us on insect cadavers (Table 3) are considered as weak pathogens and not to be the reason for the death of insects. The infected insects weakened by starving or injuring and rarely appear in natural host populations. According to Evlakhova (1974) species of genus *Aspergillus* were not typical entomopathogens and even according to Humber (1998) they were contaminants growing on cadavers after insect death. Opposite to them Bennett (2010) considered that *A. flavus* Link was capable of causing serious disease to plants, insects and vertebrates taking into account that it produced aflatoxin and was a pan-kingdom pathogen.

Mirchev (2004) found that 24.5% of mortality of *L. dispar* pupae was due to diseases caused by *Scopulariopsis brevicualis* (Sacc.) Bain, *A. flavus*, *Penicillium frecuentans* Westl., *Mucor mucedo* Fres., *M. globusus* Fischer, *Aspergillus* sp. and *Fusarium* sp.

The analyses of the found dead or diseased lepidopteran forest pests showed that except mycoses there were another reasons for the decreased population density (Table 3). We marked them as „others" and they presented 38.6% of all cases of insect death. As a whole it could be mentioned that they were with different causal agents – virus, bacteria, parasitoids or predators, but they will be object of our future study.

Bioassays with *L. dispar* larvae

The results obtained in bioassays revealed that first-second instar *L. dispar* larvae were tolerant to mycoses caused by the examined *B. bassiana* isolates 588Bb, 593Bb and 596Bb. At the 17th day after the treatments, values of cumulative daily mortality due to mycoses reached hardly to 14.44% ± 1.28, 15.56% ± 1.15 and 14.44% ± 1.26, respectively

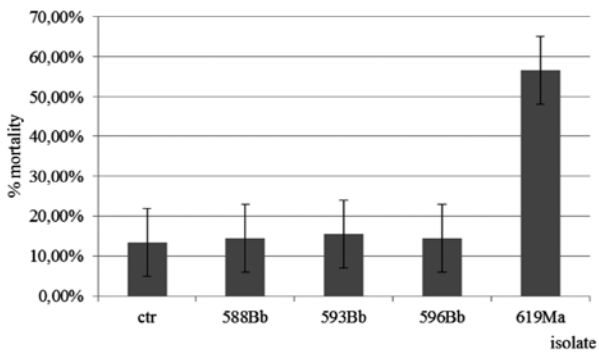

Fig. 1. Lethal effect of mycoses caused by 3 *Beauveria bassiana* isolates (588Bb, 593Bb, 596Bb) and one isolate of *Metarhizium anisopliae* (619Ma) to first-second instar *Lymantria dispar* larvae established at the 17th day after the treatment with conidial suspensions ($1x10^8$ conidia/ml)

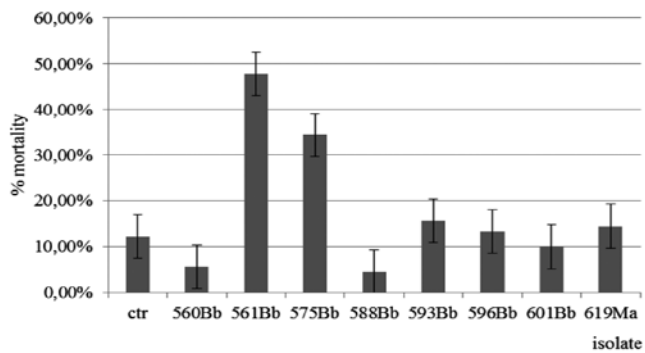

Fig. 2. Lethal effect of mycoses caused by isolates of *Beauveria bassiana* (560Bb, 561Bb, 575Bb, 588Bb, 593Bb, 596Bb, 601Bb) and *Metarhizium anisopliae* (619Ma) to third instar *Lymantria dispar* larvae established at the 17th day after the treatment with conidial suspensions ($1x10^8$ conidia/ml)

Table 4. Differences between mean lethal effects of mycoses to first-second instar *L. dispar* larvae in treatments with conidial suspensions ($1x10^8$ conidia/ml) of 4 *B. bassiana* isolates and one isolate of *M. anisopliae*

Variants compared	Mean lethal effect Group 1	Mean lethal effect Group 2	t	df	p	t separ. var.est.	df	p 2-sided	Valid N Group 1	Valid N Group 2	Std.Dv. Group 1	Std.Dv. Group 2	F-ratio variances	p variances
588Bb_L1 vs. CTR_L1	0,0732	0,0510	1,1092	32	0,2756	1,1092	30,9365	0,2759	17	17	0,0527	0,0636	1,4552	0,4614
593Bb_L1 vs. CTR_L1	0,0837	0,0510	1,7005	32	0,0987	1,7005	29,5648	0,0994	17	17	0,0473	0,0636	1,8050	0,2482
596Bb_1 vs. CTR_1	0,0876	0,0510	1,8361	32	0,0757	1,8361	30,8062	0,0760	17	17	0,0521	0,0636	1,4902	0,4337
619Ma_L1 vs. CTR_L1	0,3693*	0,0510*	6,2638*	32*	0,0000*	6,2638*	19,2124*	0,0000*	17*	17*	0,1996*	0,0636*	9,8600*	0,0000*
593Bb_L1 vs. 588Bb_L1	0,0837	0,0732	0,6095	32	0,5465	0,6095	31,6358	0,5465	17	17	0,0473	0,0527	1,2404	0,6717
596Bb_L1 vs. 588Bb_L1	0,0876	0,0732	0,8003	32	0,4294	0,8003	31,9955	0,4294	17	17	0,0521	0,0527	1,0240	0,9627
596Bb_L1 vs. 593Bb_L1	0,0876	0,0837	0,2292	32	0,8202	0,2292	31,7106	0,8202	17	17	0,0521	0,0473	1,2113	0,7061
619Ma_L1 vs. 588Bb_L1	0,3693*	0,0732*	5,9123*	32*	0,0000*	5,9123*	18,2194*	0,0000*	17*	17*	0,1996*	0,0527*	14,3484*	0,0000*
619Ma_L1 vs. 593Bb_L1	0,3693*	0,0837*	5,7396*	32*	0,0000*	5,7396*	17,7923*	0,0000*	17*	17*	0,1996*	0,0473*	17,7976*	0,0000*
619Ma_L1 vs. 596Bb_L1	0,3693*	0,0876*	5,6294*	32*	0,0000*	5,6294*	18,1678*	0,0000*	17*	17*	0,1996*	0,0521*	14,6935*	0,0000*

* difference between variants is significant

(Fig. 1). Larvae were relatively more susceptible to mycosis caused by the *M. anisopliae* isolate 619Ma. Calculated values of larval mortality established in the treatments with 619Ma were 56.67% ± 4.84.

Differences between mean lethal effects of mycoses to first-second instar *L. dispar* larvae in treatments with conidial suspensions ($1x10^8$ conidia/ml) of fungal isolates were estimated by t-test for independent samples (Table 4). Significant difference was proved at p-level<0.05 only between variant treated with conidial suspensions of 619Ma, control variant and treatments with *B. bassiana* isolates. The examined three *B. bassiana* isolates were not significantly different among each other by their lethal effect to first-second instar *L. dispar* larvae, and they were not different from the control variant as well.

The results of relative susceptibility estimation showed that the third instar *L. dispar* larvae were more tolerant to mycoses caused by the examined *B. bassiana* and *M. anisopliae* isolates than younger instars (Fig. 2). The established values of cumulative daily mortality due to mycoses were very low and only treatments with 561Bb and 575Bb caused mycoses with lethal effect 47.78% ± 4.06 and 34.44% ± 3.28, respectively.

Nevertheless the isolate 575Bb was obtained from dead *L. dispar* larva, it was with low virulence to larvae of its initial host. It seems that *L. dispar* is tolerant to mycoses caused by Hyphomycetes and especially by *B. bassiana* and *M. anisopliae* as reports about natural occurrence of these pathogens are few.

According to Polovinko *et al*. (2010), *L. dispar* larvae were less sensitive to the isolates of *B. bassiana*, which was confirmed by slow progress of the mycosis. The caterpillars of *L. dispar* died in 13–18 days after the treatment with selected 11 West Siberian isolates of the fungus *B. bassiana*. The authors considered that the isolates from a certain host were not necessarily more virulent for the same insect species.

Differences between mean lethal effects of mycoses to third instar *L. dispar* larvae in opposite to first-second instars were significantly proved at p-level<0.05 in most of the cases.

Conclusions

The presented study reveals the distribution of hyphomycete entomopathogens in natural populations of 7 lepidopteran species in Bulgarian forests. *Beauveria bassiana* was established as a dominating species affecting individuals from larval, pupal and imaginal stages. The occurrence of *B. bassiana* in *Malacosoma neustria*, *Tortrix viridana* and *Melitaea didyma* is the first record in Bulgaria.

The bioassays showed that *L. dispar* larvae were tolerant to mycoses caused by the tested *B. bassiana* isolates and *M. anisopliae*. Future investigations should be directed to search for more virulent fungal isolates and perhaps new fungal pathogens as well.

Acknowledgements: We are especially indebted to the National Science Fund of Bulgaria, Project DO-02-251/2008, and to Prof. Leellen Solter for providing gypsy moth egg masses.

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Received: 14.05.2011
Accepted: 12.06.2013