

Studies on Non-target Phyllophagous Insects in Oak Forests as Potential Hosts of *Entomophaga maimaiga* (Entomophthorales: Entomophthoraceae) in Bulgaria

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Abstract: The impact of entomopathogenic fungus *Entomophaga maimaiga* HUMBER, SHIMAZU & SOPER (Entomophthorales: Entomophthoraceae) on the complex of phyllophagous insects in oak forests in Bulgaria was studied during the period 2009-2011. From 15 populations of gypsy moth, *Lymantria dispar* (L.), i.e. six sites where *E. maimaiga* was introduced and nine sites where the pathogen occurred naturally, a total of 1499 larvae of non-target phyllophagous insects were collected. These insects belonged to 38 species of 10 families of Lepidoptera: Lycaenidae (1 species), Tortricidae (5), Pyralidae (1), Ypsolophidae (1), Geometridae (11), Noctuidae (8), Nolidae (1), Erebiidae (5), Notodontidae (1), Lasiocampidae (2) as well as to two species of Tenthredinidae (Hymenoptera). Microscopic analyses indicated no presence of *E. maimaiga* life stages in any of dead larvae. In one species, *Catocala nymphagoga* (Esper) (Lepidoptera: Erebiidae), the entomopathogenic fungi *Entomophaga aulicae* (Reich.) Humber, *Tarichium dissolvens* (Vosseler) Lakon, *Isaria farinosa* (Holmsk.) Fr., *Beauveria bassiana* (Bals.-Criv.) Vuill., *Beauveria* sp., *Conidiobolus* sp. and *Lecanicillium* sp. did cause high mortality of larvae and pupae (between 39.1 and 100%, average 61.8%). The present study represents the first record of *Tarichium dissolvens* in Bulgaria. The results of this study confirm that *E. maimaiga* is a host-specific pathogen of gypsy moth and its introduction is not dangerous for the non-target insect species in these oak forests.

Key words: *Entomophaga maimaiga*, non-target species, *Catocala nymphagoga*, *Tarichium dissolvens*, Bulgaria

Introduction

The gypsy moth, *Lymantria dispar* (L., 1758) (Lepidoptera: Erebiidae), is the most important insect pest in deciduous forests of Bulgaria and other countries of the Balkan Peninsula and Southeastern Europe where in outbreak years it caused defoliations in oak forests on an area of 200 000 – 600 000 ha (McMANUS, CSÓKA 2007).

The entomopathogenic fungus *Entomophaga maimaiga* Humber, Shimazu & Soper (Entomophthorales: Entomophthoraceae) was originally described as a fungal pathogen of *Lymantria dispar japonensis* Motschulsky, 1861, from central Honshu, Japan (SOPER *et al.* 1988; HAJEK 1999). It was intentionally introduced into USA in 1910-1911

and may or may not have been re-introduced by some unknown means in ca. 1985-1986 (HAJEK *et al.* 1995a). In 1989, the pathogen caused a strong epizootic in *L. dispar* outbreaks in many areas of north-eastern United States (ANDREADIS, WESELOH 1990). After 1989, the fungus enlarged its range following gypsy moth invasion in North America and currently occurs in 17 states of USA (HAJEK *et al.* 2005; etc.) and one province of Canada (Ontario) (HOWSE, SCARR 2002).

In 1999, *E. maimaiga* was successfully introduced into Bulgaria from USA (PILARSKA *et al.* 2000). In 2005, the pathogen caused extensive epizootics in gypsy moth populations in different regions of the country (PILARSKA *et al.* 2006). After 2005, *E. maimaiga* spread throughout nearly all areas of *L. dispar* in Bulgaria (GEORGIEV *et al.* 2011). The fungus enlarged its range and was recently reported from Georgia (KERESSELIDZE *et al.* 2011), the European part of Turkey (GEORGIEV *et al.* 2012a), Serbia (TABAKOVIĆ-TOŠIĆ *et al.* 2012), Greece, and the Former Yugoslavian Republic of Macedonia (GEORGIEV *et al.* 2012b).

E. maimaiga is a very effective pathogen at both high and low population densities of *L. dispar* and is so effective that some states in USA no longer maintain gypsy moth management programs (KERESSELIDZE *et al.* 2011). After the *E. maimaiga* introduction into Bulgaria, the outbreaks of *L. dispar* now affect only 10-20% of the area typical for previous pest infestations (GEORGIEV *et al.* 2011).

E. maimaiga is a highly host-specific pathogen affecting only *L. dispar* in its natural range (HAJEK 1999). In the USA, field studies showed that, in addition to *L. dispar*, the fungus causes low mortality in only 3 closely related species of Erebididae and single representative species from 2 other lepidopteran families (HAJEK *et al.* 1996). These results indicate that *E. maimaiga* possesses great potential for development as a biological control agent against its intended target host but in the areas where this fungus is newly introduced, it is still important to evaluate its impact on non-target native insects.

This paper reports results from the study on impact of *E. maimaiga* on non-target phyllophagous insects in oak forests in Bulgaria.

Material and Methods

The studies were conducted during the period 2009-2011 in oak forests in 15 localities in Bulgaria – six on sites where *E. maimaiga* had been intentionally introduced (Gorni Domlian, Gabrovitsa, Striama, Sadievo, Assenovo, Slavyanovo) and 9 on sites

where the pathogen had previously spread naturally into populations of gypsy moth (Skravena, Elovitsa, Spahievo, Kremen, Ravna Gora, Zvezdets, Indzhe Voyvoda, Fakia, Karlanovo). The main characteristics of the study sites (geographical coordinates, altitudes and species composition of oak stands) are described in another publication (GEORGIEV *et al.* 2013).

Biological material was collected in study plots created for investigation of *E. maimaiga* impact on *L. dispar*. In each study plot, double-layered burlap belts (about 30 cm in width) were placed on 25 oak trees over the entire circumference of the trunks at a height of 1.3 m from the ground, for collection of gypsy moth larvae.

The larvae of non-target herbivorous insects were collected 2-3 times per month from early May to late July mainly on foliage in lower parts of tree crowns and by beating oak branches with a stick. Specimens were also collected from the burlap bands.

After collection, the larvae of non-target insects were transported to the laboratory, sorted by species, and reared in groups of 10-20 on fresh oak foliage in plastic boxes (15x10x8 cm). The foliage was changed daily and the dead larvae were separated individually in Petri dishes with water-saturated filter paper disc at 20°C for 4-5 days. For a week the Petri dishes were stored at room temperature and then refrigerated at 5°C until microscopical evaluation. Microscopical analyses were made with microscopes Carl Zeiss Axiostar Plus and Carl Zeiss Jena NU 2 at 100x and 400x. Each cadaver was dissected individually and observed under light microscope for the presence of conidia or azygospores of *E. maimaiga* or for other pathogens.

The non-target insect species were identified in larval or adult stages using different keys. A part of species were identified or confirmed by Prof. Dr. Marek Turcani, Czech University of Life Sciences, Faculty of Forestry and Wood Sciences, Czech Republic and Dr. Stoyan Beshkov, National Museum of Natural History, Bulgaria. However, some lepidopteran and hymenopteran larvae were not identified and were not included in this paper.

Results

In 2009, 739 specimens of 34 non-target species were collected, reared and analyzed; in 2010, 587 specimens of 27 species; and in 2011, 173 specimens of 17 species. A total of 1499 specimens belonging to 38 species in 10 families of Lepidoptera – Lycaenidae (1 species), Tortricidae (5), Pyralidae (1), Ypsolophidae

Table 1. Non-target insects studied during the period 2009-2011 in different localities in Bulgaria

Non-target insect	Localities (Number of insects examined)	Total number
Lycaenidae		
<i>Favonius quercus</i> (L.)	Karlanovo (1)	1
Tortricidae		
<i>Aleimma loeflingiana</i> (L.)	Striama (1), Skravena (9)	10
<i>Archips xylosteana</i> (L.)	Gorni Domlian (2), Striama (16), Assenovo (6), Elovitsa (2), Spahievo (3), Zvezdets (1), Indzhe Voyvoda (5), Karlanovo (4)	39
<i>Tortrix viridana</i> L.	Gorni Domlian (13), Gabrovnitsa (32), Striama (44), Sadievo (6), Assenovo (7), Skravena (17), Elovitsa (13), Spahievo (15), Kremen (18), Ravna Gora (5), Zvezdets (8), Indzhe Voyvoda (5), Karlanovo (53)	236
<i>Ancylis</i> sp.	Striama (1)	1
<i>Archips</i> sp.	Karlanovo (21)	21
Pyralidae		
<i>Acrobasis consociella</i> (Hb.)	Elovitsa (8), Ravna Gora (32), Zvezdets (5)	45
Ypsolophidae		
<i>Ypsolopha</i> sp.	Gorni Domlian (17), Striama (11), Skravena (13)	41
Geometridae		
<i>Alsophila aescularia</i> (DEN. & SCHIFF.)	Gabrovnitsa (17)	17
<i>Alsophila aceraria</i> (DEN. & SCHIFF.)	Gabrovnitsa (7), Skravena (3)	10
<i>Agriopis aurantiaria</i> (Hb.)	Gorni Domlian (2), Striama (10), Karlanovo (14)	26
<i>Agriopis leucophaearia</i> (DEN. & SCHIFF.)	Striama (15), Skravena (5), Karlanovo (7)	27
<i>Agriopis marginaria</i> (F.)	Striama (5), Skravena (7), Karlanovo (7)	19
<i>Colotois pennaria</i> (L.)	Striama (5), Skravena (7)	12
<i>Erannis defoliaria</i> (Cl.)	Gabrovnitsa (4), Assenovo (1), Skravena (11), Elovitsa (2), Spahievo (12), Ravna Gora (5), Zvezdets (4), Indzhe Voyvoda (2), Karlanovo (16)	57
<i>Operophtera brumata</i> (L.)	Gorni Domlian (10), Gabrovnitsa (34), Skravena (13), Elovitsa (2), Spahievo (2), Kremen (2), Ravna Gora (5), Zvezdets (9), Indzhe Voyvoda (30), Karlanovo (17)	121
<i>Agriopis</i> sp.	Gorni Domlian (7), Gabrovnitsa (4), Karlanovo (5)	16
<i>Alsophila</i> sp.	Gabrovnitsa (6), Karlanovo (2)	8
<i>Biston</i> sp.	Gorni Domlian (5), Striama (1), Karlanovo (4)	10
Noctuidae		
<i>Amphipyra pyramidea</i> (L.)	Spahievo (1), Zvezdets (2)	3
<i>Anorthoa munda</i> (DEN. & SCHIFF.)	Striama (2)	2
<i>Cosmia trapezina</i> (L.)	Gabrovnitsa (2)	2
<i>Orthosia cerasi</i> (F.)	Striama (7), Karlanovo (15)	22
<i>Orthosia cruda</i> (DEN. & SCHIFF.)	Gorni Domlian (6), Gabrovnitsa (9), Striama (46), Skravena (51), Spahievo (3), Karlanovo (30)	145
<i>Orthosia miniosa</i> (DEN. & SCHIFF.)	Striama (2)	2
<i>Acronicta</i> sp.	Slavyanovo (1), Elovitsa (1)	2
<i>Orthosia</i> sp.	Gorni Domlian (14), Gabrovnitsa (71), Striama (25), Skravena (10), Kremen (1)	121
Nolidae		
<i>Bena bicolorana</i> (FUESSLY)	Skravena (3)	3

Table 1. Continued

Non-target insect	Localities (Number of insects examined)	Total number
Erebidae		
<i>Catocala nymphagoga</i> (Esp.)	Gabrovnitsa (5), Assenovo (1), Slavyanovo (68), Skravena (23), Elovitsa (16), Spahievo (1), Kremen (2), Ravna Gora (40), Indzhe Voyvoda (59), Fakia (13)	228
<i>Catocala sponsa</i> (L.)	Zvezdets (1)	1
<i>Eilema complana</i> (L.)	Gabrovnitsa (23), Gabrovnitsa (6), Elovitsa (2), Spahievo (6), Ravna Gora (25), Zvezdets (3)	65
<i>Euproctis chrysorrhoea</i> (L.)	Sadievo (34), Kremen (1)	35
<i>Lymantria monacha</i> (L.)	Elovitsa (1)	1
Notodontidae		
<i>Thaumetopoea processionea</i> (L.)	Ravna Gora (91)	91
Lasiocampidae		
<i>Eriogaster lanestris</i> (L.)	Sadievo (1)	1
<i>Malacosoma neustria</i> (L.)	Sadievo (1)	1
Tenthredinidae		
<i>Apethymus</i> sp.	Gorni Domlian (5), Gabrovnitsa (12), Striama (11), Skravena (15), Karlanovo (6)	49
<i>Periclista</i> sp.	Striama (8)	8
Total		1499

(1), Geometridae (11), Noctuidae (8), Nolidae (1), Erebidae (5), Notodontidae (1), Lasiocampidae (2) – and two species of Tenthredinidae (Hymenoptera) were studied over this entire period (Table 1).

Part of the laboratory-reared insects died in the larval or pupal stage. Mortality rate of the reared insects collected from different localities was below 5-10% except for *Catocala nymphagoga* (Esper, 1787) (Lepidoptera: Erebidae). However the mortality of *C. nymphagoga* larvae collected from different localities and reared under laboratory conditions varied in large limits – between 0 and 100%, at average value for the country of 61.8% (Table 2).

Microscopic analyses of dead larvae belonging to different species did not show presence of conidia and azygospores of *E. maimaiga*. Infections of the non-target lepidopterans caused by other fungal pathogens were found only in *C. nymphagoga*. These 'other' fungal pathogens were identified as *Entomophaga aulicae* (Reich.) Humber, *Tarichium dissolvens* (Vosseler) Lakon, *Isaria farinosa* (Holmsk.) Fr., *Beauveria bassiana* (Bals.-Criv.) Vuill., *Beauveria* sp., *Conidiobolus* sp. and a *Lecanicillium* sp. (Table 2).

Fungal pathogens were identified after termination of the experiments, and it was not possible to provide precise values for mortality of *C. nymphagoga* caused by the fungal species mentioned

above. *Entomophaga aulicae*, which is taxonomically closest to *E. maimaiga*, was found from only one dead larva of *C. nymphagoga*.

T. dissolvens, a poorly known entomophthorean fungus that produces only thick-walled resting spores, was isolated from larvae collected from Slavyanovo and reared under laboratory conditions as well as from larvae found dead on burlap bands at Slavyanovo and Skravena. This is the first report of this fungus from *C. nymphagoga* and it is a first report of this species for the Bulgarian mycota. *E. aulicae*, *I. farinosa* and *B. bassiana* have not been recorded until now from *C. nymphagoga* in Bulgaria.

Discussion

It is desirable that microbial biological control agents introduced into forest sites should have high specificity to avoid negative impact on the other (non-target) insects in the forest ecosystems.

After the recognition of *E. maimaiga* in the USA, the specificity of this pathogen was assessed in both laboratory experiments and natural conditions. Bioassays with conidial suspensions showed that *E. maimaiga* could infect species in seven out of ten lepidopteran superfamilies; with exception of *Malacosoma disstria* Hübner (Lasiocampidae) and *Manduca sexta* (L.) (Sphingidae), infections were

Table 2. Mortality of *Catocala nymphagoga* in different localities

Locality	Year	Number of larvae studied	Number of dead larvae and pupae	Mortality (%)	Fungal pathogens
Elovitsa	2009	4	0	0	-
	2010	12	10	83.3	<i>Entomophaga aulicae</i> <i>Beauveria bassiana</i> <i>Isaria farinosa</i> <i>Lecanicillium</i> sp.
Assenovo	2010	1	0	0	-
Gabrovnitsa	2010	5	0	0	-
Slavyanovo	2010	68	56	82.4	<i>Tarichium dissolvens</i> <i>Conidiobolus</i> sp.
Indze Voyvoda	2010	59	38	64.4	<i>Beauveria bassiana</i> <i>Beauveria</i> sp.
Skravena	2010	23	9	39.1	<i>Tarichium dissolvens</i> <i>Conidiobolus</i> sp.
Ravna Gora	2010	38	17	44.7	<i>Beauveria</i> sp. <i>Fusarium</i> sp.
	2011	2	1	50.0	*
Kremen	2010	1	0	0	-
	2011	1	1	100.0	*
Spahievo	2011	1	1	100.0	*
Fakia	2011	13	8	61.5	*
Total		228	141	61.8	

* Cause of mortality not determined

very low in species from all families other than Erebidae (HAJEK *et al.* 1995b). In the field, only cadavers of three *Dasychira* species (a close relative of *L. dispar*) were found to be infected by *E. maimaiga*: *D. basiflava* (Packard), *D. obliquata* (Crote & Robinson) and *D. leucophaea* (J. E. Smith) (HAJEK *et al.* 1996). According to the authors, in sites with high gypsy moth population densities and epizootics caused by *E. maimaiga*, from 1,511 larvae of 52 species in seven lepidopteran families that were collected and reared in the laboratory, only 2 individuals died from infection by *E. maimaiga*: *M. disstria* (0.3% of the studied specimens) and *Catocala ilia* (Cramer) (Erebidae) (1.0%). However, in areas with low gypsy moth density, no infected non-target specimens were collected.

The infection of gypsy moth mainly occurs on soil litter at the base of the stems that serves as the main reservoir for the azygospores of *E. maimaiga* (HAJEK *et al.* 1998). In the US, experiments to rear lepidopteran species around the base of the stems of *Quercus rubra* L. found only one specimen of Gelechiidae (1.2% of the surveyed larvae) and a specimen of *Sunira bicolorago* (Gn.) (Noctuidae) (5.0%), were infected with *E. maimaiga* when, in the same sites, *L. dispar* the infection level reached 37.3% (HAJEK *et al.* 2000). These results confirm *E.*

maimaiga to be a highly specific pathogen of gypsy moth and justify the increasingly wider use of this fungus in programs for biological control of gypsy moth.

With regard to the fungal pathogens from *C. nymphagoga* found during this study, we should mention that only *Tarichium dissolvens* is new for the Bulgarian mycota; this fungus has been reported previously as a pathogen of *M. disstria* (SOPER, MACLEOD 1981). The other six species have been reported previously from different lepidopteran hosts in Bulgaria but have never been found to affect *C. nymphagoga*. *C. nymphagoga* has never been registered as an economically important pest in Bulgarian forests, which is probably due to the impact of established fungal pathogens on its population density.

In conclusion we should note that the results in Bulgaria confirm the high species specificity of *E. maimaiga* and characterize this pathogen as harmless for the general complex of phyllophagous insects in oak forests. *E. maimaiga* has a high virulence for gypsy moth and can form the basis for the development of integrated system for the control of the pest. Since the introduction of *E. maimaiga* in Bulgaria there have been few serious outbreaks of *L. dispar*, and the use of insecticides is already being reduced. The presence and activity of *E. mai-*

maimaiga has been very important for conservation of biodiversity in oak ecosystems that harbor many lepidopteran species with high conservation value but that are vulnerable to the effects of applications of entomopathogenic preparations (e.g., of *Bacillus*

thuringiensis and *Saccharopolyspora spinosa*) or of insecticides that may be toxic to such non-target lepidopterans.

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