UDC: 632.78:632.5

Topola Poplar SINCE 1957

Original scientific paper

Lymantria dispar Mortality in Pupal Stage Caused by *Entomophaga maimaiga* in Bulgaria and Serbia

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Abstract: The impact of introduced fungal pathogen *Entomophaga maimaiga* on population density of *Lymantria dispar* (L.) was studied in Bulgaria and Serbia during the period 2009-2014. In many localities, strong mortality was observed not only during the larval development but also in the pupal stage of the host. The average annual gypsy moth mortality caused by *E. maimaiga* in Bulgaria varied between 66.5% and 86.8%, and in Serbia – between 62.8% and 98.8%. The pupal mortality in Bulgaria and Serbia varied between 11.7-33.1% and 0.4-6.3%, respectively. The analysis of the biological material showed that the number of dead pupae was considerably high, in spite of the established small amount of the pathogen's azigospores. The number of azigospores in *L. dispar* dead pupae in three studied localities in Serbia varies strongly (12-30 per a view field), but the average values are very close (2.33-2.90).

Keywords: *Lymantria dispar*, *Entomophaga maimaiga*, host mortality, pupal stage, Bulgaria, Serbia.

1. Introduction

The gypsy moth, *Lymantria dispar* (Linnaeus, 1758) (Lepidoptera: Erebidae), is one of the most harmfulness insect pests in forest ecosystems of Europe, Asia, Japan and North Africa. Widely polyphagous, it is trophycally connected with more than 300 deciduous and coniferous tree and shrub species, mainly preferring oaks (*Quercus* spp.) and poplars (*Populus* spp.). In 1868, the gypsy moth was accidentally introduced from Europe to North America. In the new habitats, many introductions of parasitoids and pathogens of the pest have been conducted from its native range (Europe and Asia), including entomopathogenic fungus *Entomophaga maimaiga* Humber, Shimazu & Soper (Entomophthorales: Entomophthoraceae) (Hajek et al. 1995). Gypsy moth epizootics caused by *E. maimaiga* were first observed in 1989 in seven Northwestern states (Andreadis and Weseloh, 1990). Since that time, the fungus has been spread through most of the range of *L. dispar* in US and Canada, and is responsible for collapses of many local gypsy moth populations (Tobin and Hajek, 2012).

In 1999, *E. maimaiga* was successfully introduced in Europe, in the region of Central Bulgaria (Pilarska et al. 2000). The first strong epizootics caused by the fungus were observed in 2005 in different areas of Northern and Southern Bulgaria (Pilarska et al. 2006). The fungus enlarged its range in many localities of the country through naturally spreading or new introductions (Georgiev et al. 2013). In 2011, *E. maimaiga* was established in Serbia (Tabaković-Tošić et al. 2012) and European part of Turkey (Georgiev et al., 2012). In 2012, *E. maimaiga* penetrated in Greece, Macedonia (Georgieva et al. 2013), Croatia (Hrašovec et al. 2013) and Romania (Netoiu et al. 2016), and in 2013 – in Bosnia and Herzegovina (Milotić et al. 2015), Hungary (Csóka et al. 2014) and Slovakia (Zubrik et al. 2014).

E. maimaiga is a host specific, high virulent and very effective regulator of gypsy moth density and has great potential as a biological control agent. The life cycle of the pathogen is well synchronized with host larval development. It produces two types of spores: conidia and resting (azygospores). Conidia are formed mainly in young (early-instar) larvae, and mortality of the host occurs in tree crowns. Azygospores are formed in older (late-instar) larvae, and mortality occurs on tree stems. It is known that a part of infected gypsy moth larvae die in pupal stage (Hajek, 1999).

In this paper we report the results of pupal mortality of *L. dispar* caused by *E. maimaiga* in host populations in Bulgaria and Serbia.

2. Material and Methods

The studies were conducted in 2011-2014 in 34 localities of *L. dispar* in which epizootics caused by *E. maimaiga* occurred in Bulgaria and Serbia. The main characteristics of studied localities and forest stands are pointed in the Tables 1 and 2.

Table 1. Main characteristics of studied areas in Bulgaria.

Forest Enterprise*	Locality**	Geographical coordinates	Altitude (m a.s.l.)	Stand composition: Tree species	Stand composition: %	Age (year)
SFE Gorna Oryahovitsa	Asenovo vill.	N43°17'41.7" E26°04'50.0"	401	*Quercus cerris*	100	65
SFE Govezhda	Elovitsa vill.	N43°19'51.0" E23°00'14.8"	345	*Carpinus orientalis* *Quercus cerris*	100 Single trees	50
SFE Haskovo	Spahievo vill.	N41°52'51.4" E25°19'29.7"	451	*Quercus frainetto*	100	55
SFE Kirkovo	Kremen vill.	N41°17'08.0" E25°19'52.1"	474	*Quercus frainetto* *Quercus petraea*	70 30	60
SFE Nova Zagora	Sadievo vill.	N42°31'46.9" E26°08'54.0"	151	*Quercus robur*	100	50
SHE Cherni Lom (Popovo)	Slavyanovo vill.	N43°17'05.4" E26°08'03.1"	345	*Quercus cerris*	100	65
SHE Staro Oryahovo	Obzor vill.	N42°47'32.8" E27°52'32.8"	97	*Quercus cerris* *Quercus frainetto*	60 40	50
	Ravna gora vill.	N43°02'13.0" E27°49'56.3"	40	*Quercus cerris* *Quercus pubescens*	60 40	50
	Solnik vill.	N42°54'12.9" E27°42'56.8"	205	*Quercus frainetto* *Quercus cerris*	60 40	80
SFE Sredets	Fakia vill.	N42°13'25.7" E27°08'05.1"	362	*Quercus frainetto* *Quercus cerris* *Fraxinus ornus*	70 20 10	40
SFE Targovishte	Dalgach vill.	N43°12'57.9" E26°42'28.7"	193	*Quercus rubra* *Tilia platyphyllos*	80 20	35
SFE Zvezdets	Indzhe voyvoda vill.	N42°13'17.7" E27°27'00.4"	299	*Quercus cerris* *Quercus frainetto*	60 40	40
	Zvezdets vill.	N42°07'04.1" E27°24'07.2"	336	*Quercus petraea* *Quercus frainetto* *Quercus cerris*	90 10 Single trees	75

* SFE – State Forest Enterprise; SHE – State Hunting Enterprise; PE - Public Enterprise; FE – Forest Estate

** FA – Forest Administration

Table 2. Main characteristics of studied areas in Serbia.

Forest Enterprise*	Locality**	Geographical coordinates	Altitude (m a.s.l.)	Stand composition: Tree species	%	Age (year)
PE Srbijašume FE Beograd	Borački Gaj vill. (FA Lipovica)	N44°32' E20°21'	120-170	*Quercus cerris* *Quercus frainetto*	80 20	65
Diocese of Valjevo–Monastery forests	Bogovađa	N44°19' E20°11'	140-190	*Quercus cerris* *Carpinus betulus* *Quercus frainetto* or *Tilia argentea*	60 30 10	82
PE Srbijašume FE Boranja Loznica	Istočna Boranja (FA Krupanj)	N44°22'41.28" E19°21'40.19"	423	*Fagus sylvatica*	100	100
PE Srbijašume FE Timočke šume Boljevac	Brestovac vill. (FA Bor)	N44°02'21.24" E22°06'22.35"	400	*Quercus cerris*	90	65
	Urovica vill. (FA Negotin)	N44°24'51.38" E22°21' 08.87"	180	*Quercus cerris*	90	65
PE NP Đerdap	Monte Miroč and Crni Vrh (Reon Donji Milanovac)	N44°35'28" E22°16'49" N44°34'13" E21°53'47"	560 620	*Fagus sylvatica* *Fagus sylvatica*	100 100	90 90
PE Srbijašume FE Rasina Kruševac	Srndaljska reka (FA Kruševac)	N43°25'50.28" E21°29'14.82"	593	*Fagus sylvatica*	100	90
	Žunjačko Batotske planine (FA Brus)	N43°19'56.31" E21°11'04.35"	556	*Fagus sylvatica*	100	90
PE Srbijašume FE Toplica Kuršumlija	Mali Jastrebac (FA Prokuplje)	N43°20'32.60" E21°35'55.10"	537	*Fagus sylvatica*	100	80
	Monte Javorac (FA Blace)	N43°18'04.98" E21°11'18.18"	572	*Fagus sylvatica*	100	85
	Dobri Do (FA Kuršumlija)	N42°53'10.89" E21°23'41.42"	859	*Fagus sylvatica*	100	100
PE Šuma Goč Vrnjačka Banja	Gračac	N43°35'11.76" E20°50'15.40"	559	*Fagus sylvatica*	100	100
	Monte Goč	N43°33'03.36" E20°55'16.03"	889	*Fagus sylvatica*	90	90
PE Srbijašume FE Stolovi Kraljevo	Gledićke planine (FA Kraljevo)	N43°46'49.07" E20°55'33.39"	590	*Fagus sylvatica*	100	90
PE Srbijašume FE Severni Kučaj Kučevo	Monte Deli Jovan (FA Majdanpek)	N44°13'28" E22°15'09"	659	*Fagus sylvatica*	100	80
	Lješnica vill. (FA Kučevo)	N44°29'58.00" E21°39'54.14"	436	*Fagus sylvatica*	90	80
PE Srbijašume FE Kragujevac	Knić–Pajsijević vill. (FA Kragujevac)	N43°52'39.37" E20°42'25.81"	537	*Quercus cerris* *Quercus petraea*	50 50	65
PE Srbijašume FE Šuma Leskovac	Monte Vučje (FA Vučje)	N42°50'26.63" E21°53'19.99"	546	*Fagus sylvatica*	95	90
	Miroševce vill. (FA Leskovac)	N42°51'56" E21°50'21"	285	*Quercus cerris*	90	65
PE Srbijašume FE Niš	Monte Rtanj (FA Sokobanja)	N43°46'34" E21°53'36"	623	*Fagus sylvatica*	100	90

* SFE – State Forest Enterprise; SHE – State Hunting Enterprise; PE - Public Enterprise; FE – Forest Estate
** FA – Forest Administration

In order to collect biological material, double-layered burlap bands (about 30 cm in width) were placed on 25 oak trees in each study site. The bands surrounded the tree trunks at a height of 1.3 m from the ground. Larvae of *L. dispar* were collected from the bands, which they use as resting sites, 2-3 times a month from early May to late July. Collected larvae were returned to the laboratory and reared in groups of 10-20 on fresh oak foliage in plastic boxes (15 × 10 × 8 cm). The foliage was changed daily and dead larvae were separated individually in Petri dishes with water-saturated filter paper disc at 20°C for 4-5 days. The pupated specimens were also separated individually in Petri dishes for examination.

The Petri dishes with dead *L. dispar* larvae and pupae were stored for a week at room temperature and then refrigerated at 5°C until microscopical evaluation. Microscopical analyses were made at magnification ×100, ×125 and ×400. Each cadaver of dead larva or pupa was dissected individually and observed under light microscope for the presence of conidia or azygospores of *E. maimaiga*. The number of *E. maimaiga* resting spores in dead pupae was calculated in three sites in Serbia on the base of three samples utilizing polyester micrometer cover slips (18×18 mm). Microscopic analyses were made with a microscope NU 2 at magnification ×125.

3. Results

The mortality of *L. dispar* caused by *E. maimaiga* in different localities in Bulgaria and Serbia varied between 42.1 and 100.0% (Tables 3 and 4).

Table 3. *Lymantria dispar* mortality in Bulgaria.

Year	Locality	Studied larvae and pupae, N	Mortality (%)		
			Larval	Pupal	Total
2009	Elovitsa	32	68.8	28.1	96.9
	Kirkovo	73	52.1	31.5	83.6
	Ravna gora	205	53.2	17.5	70.7
	Sadievo	100	53.0	30.0	83.0
	Spahievo	261	23.0	49.4	72.4
	Zvezdets	146	58.9	29.5	88.4
2010	Asenovo	36	66.7	16.6	83.3
	Elovitsa	46	41.3	15.2	56.5
	Kirkovo	19	26.3	15.8	42.1
	Ravna gora	148	64.9	9.4	74.3
	Spahievo	42	59.5	9.5	69.0
	Zvezdets	54	55.6	22.2	77.8
2011	Elovitsa	5	0.0	80.0	80.0
	Dalgach	53	100.0	0.0	100.0
	Fakia	273	57.5	23.8	81.3
	Indzhe voyvoda	43	72.1	20.9	93.0
	Obzor	108	28.7	49.1	77.8
	Kirkovo	333	73.0	19.5	92.5
	Slavyanovo	32	90.6	3.1	93.7
	Solnik	89	92.1	2.3	94.4
	Spahievo	147	52.4	15.6	68.0
	Ravna gora	158	73.4	12.7	86.1
	Zvezdets	434	44.7	45.9	90.6

In Bulgaria, the larval mortality of gypsy moth varied between 0% (Elovitsa, 2011) and 100% (Dalgach, 2011). On the other hand, in above mentioned localities the pupal mortality reached up highest and lowest values in this year – 80% and 0%, respectively.

In Serbia, the larval mortality of *L. dispar* varied between 82.9% (Monte Javorac, 2013) and 100% (Gračac and Monte Deli Jovan, 2013; Monte Rogot, Miroševce and Monte Rtanj, 2014). The pulal mortality was relatively low and varied between 0% in six localities (Gračac, Monte Goč, Monte Deli Jovan, 2013; Monte Rogot, Miroševce and Monte Rtanj, 2014) and 6.9% (Beograd, 2011) (Table 4).

The average annual gypsy moth mortality caused by *E. maimaiga* in three studied years in Bulgaria varied between 66.5% (2010) and 86.8% (2011) (Figure 1). The larval and pupal mortality varied between 45.0-60.5% and 11.7-33.1%, respectively.

Table 4. *Lymantria dispar* mortality in Serbia.

Year	Locality	Studied larvae and pupae, N	Mortality (%)		
			Larval	Pupal	Total
2011	Beograd	532	85.0	6.9	91.9
	Valjevo	321	92.5	5.3	97.8
2012	Negotin	416	94.0	4.1	98.1
	Monte Miroč and Crni Vrh	536	91.8	5.0	96.8
2013	Srndaljska reka	721	96.8	2.1	98.9
	Žunjačko Batotske planine	1347	88.7	8.4	97.1
	Mali Jastrebac	271	94.5	2.2	96.7
	Monte Javorac	532	82.9	3.2	86.1
	Dobri Do	931	97.8	2.2	100.0
	Gračac	801	100.0	0.0	100.0
	Monte Goč	395	96.2	0.0	96.2
	Gledićke planine	877	86.7	9.7	96.4
	Lješnica	222	90.5	4.9	95.4
	Monte Deli Jovan	109	100.0	0.0	100.0
	Majdanpek	434	94.5	2.3	96.8
	Monte Vučje	298	98.0	2.0	100.0
	Istočna Boranja	211	94.1	5.9	100.0
2014	Monte Rogot	143	100.0	0.0	100.0
	Brestovac	117	93.2	5.1	98.3
	Miroševce	213	100.0	0.0	100.0
	Monte Rtanj	831	100.0	0.0	100.0

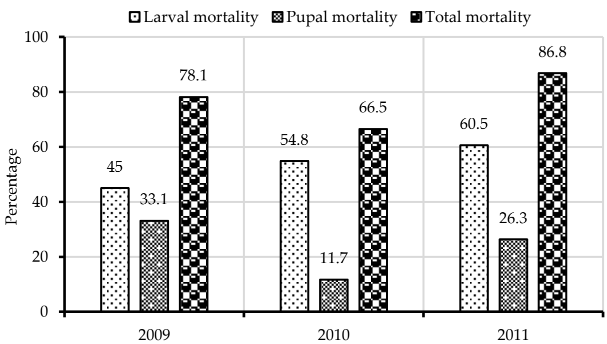

Figure 1. Average annual mortality of *Lymantria dispar* caused by *Entomophaga maimaiga* in Bulgaria.

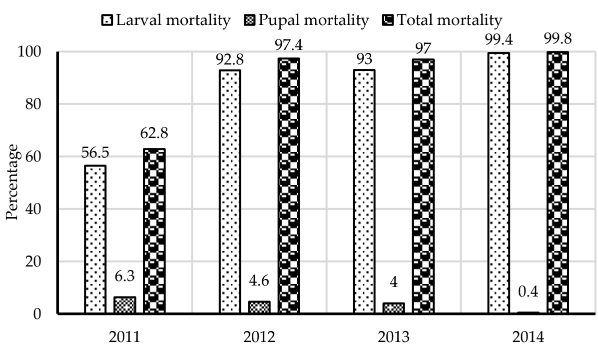

Figure 2. Average annual mortality of *Lymantria dispar* caused by *Entomophaga maimaiga* in Serbia

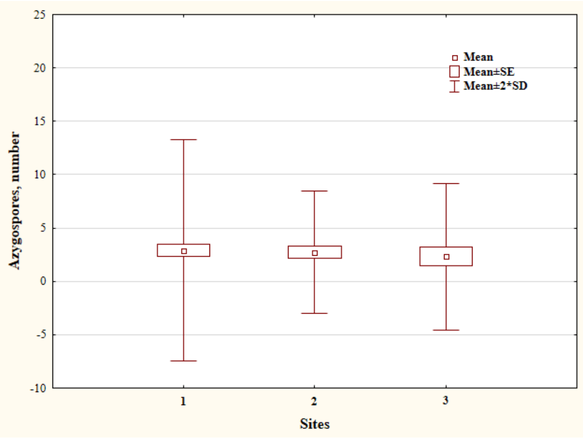

Figure 3. Density of *Entomophaga maimaiga* azygospores extracted from *Lymantria dispar* dead pupae in 2013 in Serbia: 1 - Srndaljska reka (N=83); 2 - Mali Jastrebac (N=26); 3 - Monte Vučje (N=15)

In Serbia, the average annual mortality varied between 62.8% (2011) and 98.8% (2014) (Figure 2). The larval mortality in four studied years was 56.5-99.4%, and pupal one – 0.4-6.3%.

The number of azigospores in *L. dispar* dead pupae in three studied localities in Serbia varies strongly (12-30 per a view field), but the average values are very close (2.33-2.90 per a view field) (Figure 3).

4. Discussion

It is noteworthy, that the results of *L. dispar* mortality caused by *E. maimaiga* in Bulgaria and Serbia differ significantly. For example, lower total mortality rates (66.5-86.8%) were reported in Bulgaria, compared to Serbia (62.8-99.8%). These differences are even greater for pupal mortality – 11.7-33.1% in Bulgaria and only 0.4-6.3% in Serbia. Undoubtedly, the reason for this phenomenon is different methodological approach to data reporting – in this paper, there is no data about many field epizootics in gypsy moth populations in Bulgaria, where the larval mortality rates were, as a rule 100%, and the host could not reach the pupal stage. These include mass epizootics in 2005 (Pilarska et al. 2006), epizootics as a result of artificially introductions in 2008-2011 (Georgiev et al. 2013) and the spontaneous epizootics that occurred in Kirkovo region in 2013-2014 (Georgiev et al. 2014). The use of these data would, on the one hand, lead to an increase in the values of larval and total mortality of gypsy moth, and a reduction in pupal mortality rates, on the other hand.

It is well known that the environmental conditions are of great importance for the spread of *E. maimaiga* and and its impact on the host. Production and density of conidia are higher in the areas with higher relative humidity, often occurred after the rain (Hajek et al. 1990; Hajek et al. 1999). Spore production and release was higher at 95-100% relative humidity, and only limited spore production was seen at 50-70% relative humidity. Conidia required also free water for germination (Hajek et al. 1990). Humid spring time in late April and May is positively associated with infections by *E. maimaiga*. Under favorable conditions, the conidial infections affect early-instar gypsy moth larvae and cause a massive mortality prior to causing defoliation on the host plants. In this case, the host died long time before pupating.

Infection with germ conidia of resting spores can also occur later when late-instar gypsy moth larvae descend the host trees to rest during the day on tree trunks or on forest litter. Epizootics usually occur near the end of larval development, where tree trunks and branches are covered with dead larvae. It could be noted that the production of azigospores is also positively associated with temperature and moisture level (Shimazu, 1987). Under unfavorable conditions and low extensiveness of fungal infection, pupation of infected late-instar *L. dispar* larvae should be expected with subsequent mortality in the pupal stage.

In conclusion, the pupal mortality of *L. dispar* caused by the fungal pathogen is of great importance for health status assessment of pest populations. *E. maimaiga* can be regarded as a good alternative to the use of chemical and bacterial insecticides for pest control, which is of great importance for the quality of human environment and biodiversity conservation in forest ecosystems.

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