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RESEARCH ARTICLE

Biological control of gypsy moth (*Lymantria dispar*) by the entomopathogenic fungus *Entomophaga maimaiga* in Bulgaria in 2021

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Abstract

A biological control programme against gypsy moth (*Lymantria dispar*) populations was carried out in 2021 via the inoculative release of the entomopathogenic fungus *Entomophaga maimaiga* on the territory of four State Forest Enterprises: Municipal Enterprise (ME) 'Management of Municipal Forests, Agriculture and Forestry', Nessebar; State Game Enterprises (SGE) Nessebar and Balchik; State Forestry (SF) Vidin. The pathogen was released during the period 15-26.03.2021 in 34 localities – five in ME Nessebar, eight in SGE Nessebar, ten in SGE Balchik and eleven in SF Vidin. The average number of gypsy moth population density in the locations of introduction was relatively high, ranging between 0.4-15.9 egg masses/tree in the area of SGE Balchik and 11.9-65.0 egg masses/tree in the area of ME Nessebar. The average mortality of young gypsy moth caterpillars (first-third instar) due to *E. maimaiga* varied between 2.6% (SGE Balchik) and 13.0% (SF Vidin), and of caterpillars in later fourth-sixth instar – between 20.7% (SF Vidin) and 52.4% (ME Nessebar). The lowest overall mortality of the gypsy moth caterpillars due to *E. maimaiga* was in the region of SGE Balchik (26.1%), followed by SF Vidin (33.7%), SGE Nessebar (48.5%) and ME Nessebar (55.9%). As a result of the release, the severe gypsy moth outbreaks in the region of Nessebar were significantly suppressed. There is a high number of *E. maimaiga* resting spores remaining in the surface layers of the soil in the other two areas (Vidin and Balchik). They have the potential to suppress *L. dispar* attacks in years to follow.

Keywords
biological control, *Lymantria dispar*, *Entomophaga maimaiga*, release

Introduction

Gypsy moth (*Lymantria dispar* L., Lepidoptera: Erebidae) is the most important defoliator in the deciduous forests of Europe, Asia and North Africa. Soon after its accidental introduction into North America in 1869, the gypsy moth started its spread as an alien invasive species, causing severe defoliation and shade trees due to the absence of natural enemies (Hajek, 2007). In Bulgaria, during severe outbreaks, *L. dispar* defoliates 110-370 thousand ha of forest deciduous stands (Georgiev et al., 2013).

The entomopathogenic fungus *Entomophaga maimaiga* Humber, Shimazu and Soper (Entomophthorales: Entomophthoraceae) was established as a species-specific pathogen of *Lymantria dispar japonensis* Motschulsky in Japan, where it regularly causes epizootics. Some introductions of the pathogen into the United States were carried-out in 1910-1911 and 1985-1986. In 1989 the pathogen was identified in gypsy moth epizootic in seven north-eastern states (Andreadis, Weseloh, 1990). Since that time, *E. maimaiga* has expanded its range by natural spread and artificial introductions in new localities of *L. dispar* (Solter, Hajek, 2009). Inoculative releases of *E. maimaiga* accelerate the spread of the pathogen and help to reduce the impact of initial and future outbreaks of the pest (Hajek et al., 2021).

In 1999, *E. maimaiga* was successfully introduced into two localities of *L. dispar* in Bulgaria (Pilarska et al., 2000). Biological material with cadavers infected by the fungus were sent from the USA. Several years after the introductions, the first severe fungal epizootics were registered in forest stands located at long distances from the places of introductions (Pilarska et al., 2006). During the period 2008-2010, all outbreaks of the gypsy moth in the country were suppressed by *E. maimaiga* releases in over ten localities of the pest (Georgiev et al., 2012, 2013; 2020). Since then, the pathogen has expanded its range by natural spread, suppressing the pest's calamity in the Northwestern Bulgaria (Georgiev et al., 2014a). In 2013-2014, a new release of *E. maimaiga* was performed in the area of SFE Kirkovo where severe mortality of gypsy moth population was achieved (Georgiev et al., 2014b).

This study presents the results of an applied biological control programme by *E. maimaiga* release to suppress the gypsy moth outbreaks in four locations in Bulgaria.

Material and methods

Attacks of the gypsy moth (Lymantria dispar) in 2021

In 2021, the attacks of the gypsy moth (*Lymantria dispar*) on the territory of four State Forest Enterprises: Municipal Enterprise (ME) 'Management of Municipal Forests, Agriculture and Forestry' Nessebar, State Game Enterprises (SGE) Nessebar and Balchik, State Forestry (SF) Vidin, covered a total of 6810.9 ha of deciduous forests (Table 1). Of these, 5832.0 ha were severely infested and intended for biological control by release of *Entomophaga maimaiga*.

Table 1. Attack of *Lymantria dispar* and severe infested forests intended for biological control

State Forest Enterprises	Degree of attacked areas, ha			Date of *E. maimaiga* release
	Slight and medium	Severe	Total	
ME Nessebar	263.0	2419.5	2682.5	15.03.2021
SGE Nessebar	350.0	1041.2	1391.2	16.03.2021
SGE Balchik	3.8	43.2	47.0	17.03.2021
SF Vidin	362.1	2328.1	2690.2	26.03.2021
Total	**978.9**	**5832.0**	**6810.9**	

The gypsy moth attacks on the territory of ME Nessebar, SGE Nessebar and SF Vidin were in natural oak forests, and on the territory of SGE Balchik – both in natural deciduous stands (the Rusalka Resort) and in field protection belts of *Quercus cerris*.

Release of Entomophaga maimaiga

The fungal pathogen was introduced in the period 15-26.03.2021 (Table 1) in 34 sample plots: five in ME Nessebar, eight in SGE Nessebar, ten in SGE Balchik (Eastern Bulgaria) and eleven in SF Vidin (Northwestern Bulgaria) (Table 2).

The biological material used for inoculations included caterpillar cadavers infected by *E. maimaiga*, collected from the tree trunks during the epizootic in 2014 in the territory of SF Kirkovo. The biological material was stored in the region of Sofia in wooden crates, filled with soil to a depth of 20-30 cm.

In 2021, the inoculum (soil mixed with infected cadavers) was released around the base of the five stems in all sample plots, by the methodology described in a previous study (Georgiev et al., 2013).

Assessment of the effect of the biological control

The effect of *E. maimaiga* on the rate of mortality in gypsy moth populations was reported by: assessment of the average number of egg masses/tree during the spring and autumn seasons of 2021; collecting gypsy moth caterpillars and bringing them to laboratory where microscopic analyses of dead caterpillars were conducted; field examination for the presence of dead caterpillars on tree trunks.

The assessment of *L. dispar* egg masses in the spring (May 2021) was performed during the release of *E. maimaiga* in the sample plots. The number of egg masses of the new generation of gypsy moth was recorded in the autumn, at the end of September and in October, in 20 locations of the fungus release: ME Nessebar (3 places); SGE Nessebar (4); SGE Balchik (7) and SF Vidin (6).

Table 2. Geographic coordinates of the locations where *Entomophaga maimaiga* was released and number of *Lymantria dispar* egg masses/tree

Locality	Coordinates		Altitude, m a.s.l.	Number of egg masses/tree		
	N	E		Min	Max	Average
ME Nessebar						
300-b	42°47'48.8"	27°51'31.7"	200	1	40	14.4
405-d	42°43'50.3"	27°52'46.6"	250	1	42	11.9
408-g1	42°43'23.4"	27°53'01.6"	150	35	122	65.0
405-g	42°43'21.2"	27°52'22.4"	300	6	89	37.6
402-d	42°43'34.4"	27°52'07.8"	60	14	88	50.0
SGE Nessebar						
353-a	42°46'30.7"	27°45'02.8"	300	0	19	18.3
375-g	42°45'23.7"	27°45'45.3"	400	7	72	28.9
376-g	42°45'28.8"	27°46'11.8"	400	5	85	41.5
378-e	42°45'21.0"	27°46'40.6"	400	17	94	50.8
378-k	42°45'23.1"	27°46'41.9"	350	16	143	64.7
437-a1	42°44'31.7"	27°47'49.8"	250	10	108	47.5
437-a2	42°44'55.9"	27°47'28.0"	330	7	75	36.1
438-g	42°44'05.9"	27°48'07.7"	300	10	50	26.9
SGE Balchik						
34-к	43°25'16.0"	28°31'18.7"	50	0	8	2.7
34-k	43°25'05.7"	28°31'03.3"	50	0	12	4.2
34-1	43°25'00.5"	28°30'53.6"	50	0	2	0.4
2339-b	43°27'17.2"	28°26'32.1"	100	0	24	8.9
2315-a	43°28'52.0"	28°26'34.5"	100	0	14	4.3
2327-a	43°30'32.1"	28°27'43.3"	100	0	27	4.7
2327-a	43°30'28.8"	28°27'39.3"	100	0	27	4.1
2302-a	43°29'23.3"	28°23'11.3"	100	0	48	15.5
2302-a	43°29'11.0"	28°22'24.6"	100	2	36	15.9
2343-a	43°28'38.3"	28°22'37.1"	100	0	32	4.4
SF Vidin						
261-a	43°52'57.1"	22°42'48.6"	200	2	72	29.0
274-i	43°51'36.4"	22°42'57.2"	200	3	42	14.2
277-m	43°52'58.5"	22°43'18.8"	150	0	0	0
280-b	43°52'05.9"	22°43'49.4"	200	0	32	6.2
281-b	43°52'43.4"	22°44'25.3"	200	0	12	2.5
285-a	43°53'22.8"	22°43'55.4"	150	0	0	0
290-p	43°52'56.1"	22°44'51.8"	150	0	5	0.6
312-z	43°49'00.7"	22°44'18.9"	200	0	13	3.1
316-a	43°47'47.9"	22°44'04.7"	200	1	15	6.3
317-z	43°47'39.3"	22°43'39.9"	200	1	21	6.9
321-f	43°46'42.2"	22°44'00.8"	200	0	4	1.2

Analysis of biological material for E. maimaiga infection

Microscopic analyses of dead caterpillars were performed at the Laboratory of phytopathology in Forest Research Institute, Sofia, with a Zeiss NU-2 light microscope at 125× magnification. Identification of the *E. maimaiga* infection was conducted by the presence of mycelium, conidia or resting spores (azygospores) of the pathogen.

Results

Average density of Lymantria dispar at the locations of E. maimaiga releases

The population density of *Lymantria dispar* assessed at the locations of releases was considerably higher, with maximum value of 143 egg masses in SGE Nessebar (Table 1). The average number of egg masses varied between 11.9 and 65.0 per tree in the area of ME Nessebar (Fig. 1A), 18.3-64.7 – in SGE Nessebar (Fig. 1B), 0.4-15.9 – in SGE Balchik (Fig. 1C) and 0-29.0 in SF Vidin (Fig. 1D).

Mortality of Lymantria dispar caterpillars in the sample plots

A total of 1695 gypsy moth caterpillars collected from ten sample plots (Table 3), were analysed in laboratory conditions. The average mortality due to the infection with the pathogen *E. maimaiga* in young larvae (first-third instar) was assessed with the highest degree in the sample plots of SF Vidin (13.0%) and the lowest – in SGE Balchik (2.6%) (Table 3). In the first collection, the average mortality of caterpillars in fourth to sixth instars, ranged between 20.7% (SF Vidin) and 52.4% (ME Nessebar) (Table 3).

The total average mortality of the gypsy moth populations due to the infection with the pathogen *E. maimaiga* was the lowest in the field protection forest belts in the area of

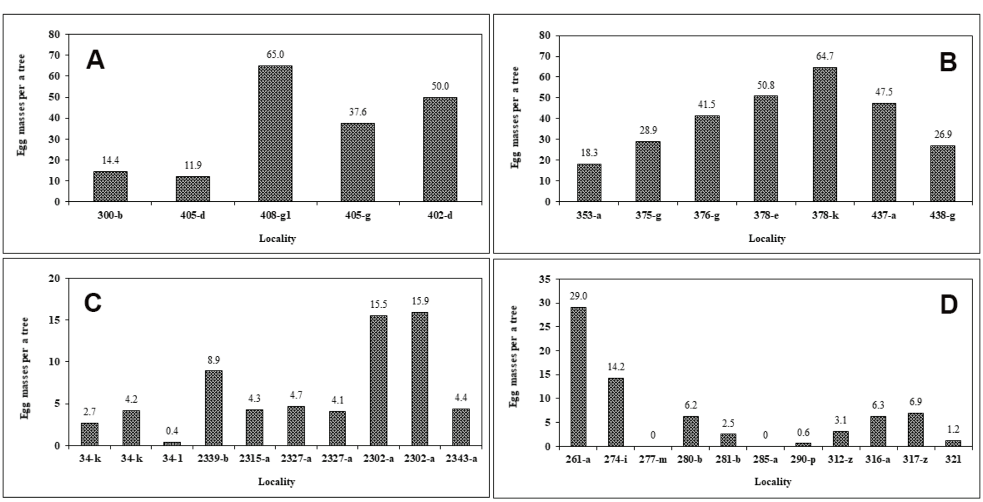

Figure 1. Number of *Lymantria dispar* in the locations of releases: A – ME Nessebar; B – SGE Nessebar; SGE Balchik; SF Vidin.

Table 3. Mortality of *Lymantria dispar* due to *Entomophaga maimaiga* in the sample plots

Locality	Studied larvae, N	Mortality					
		First – third instar		Fourth – Sixth instar		Total	
		N	%	N	%	N	%
ME Nessebar							
402-d	207	8	3.9	116	56.0	124	59.9
408-g1	165	5	3.0	79	47.9	84	50.9
Average	372	13	3.5	195	52.4	208	55.9
SGE Nessebar							
353-a	142	4	2.8	57	40.1	61	42.9
376-g	89	2	2.2	41	46.1	43	48.3
378-k	113	5	4.4	58	51.3	63	55.7
Average	344	11	3.2	156	45.3	167	48.5
SGE Balchik							
34-k	99	4	4.0	24	24.2	28	282
2339-b	135	2	1.5	31	23.0	33	24.5
Average	234	6	2.6	55	23.5	61	26.1
SF Vidin							
261	313	35	11.2	57	18.2	92	29.4
312	198	31	15.7	41	20.7	72	36.4
316	234	31	13.2	56	23.9	87	37.2
Average	745	97	13.0	154	20.7	251	33.7

SGE Balchik (26.1%). The highest pathogen efficacy was observed in the region of ME Nessebar (55.9%), followed by SGE Nessebar (48.5%) and SF Vidin (33.7%) (Table 3).

In addition to the mortality of young gypsy moth larvae caused by *E. maimaiga*, infestations of *Cotesia melanoscela* (Ratzeburg, 1984) (Fig. 2A) and *Protapanteles liparidis* (Bouché, 1834) (Hymenoptera: Braconidae) were also reported in the studied plots. The average values of parasitism of both species varied between 9.1% (SF Vidin) and 15.2% (ME Nessebar).

The lack of rainfalls in May 2021 (scarce rainfall occurred only in the region of Vidin), reflected on the low mortality rates of gypsy moth young larvae in the sample plots in the Eastern Bulgaria. Completely defoliated trees (due to the high population density of *L. dispar*) were observed in these areas. In June, the successful infection of the larvae with *E. maimaiga* positively correlated with the large amounts of rainfall that occurred during the month. Field observations in the second half of June showed that there were plenty of dead third-forth instar larvae on the ground and in shrubs under tree crowns (Fig. 2B). Analysis of the biological material showed that the cadavers of gypsy moths contained resting spores of *E. maimaiga* – evidence that the mortality was due to the pathogen. At the same period, the first dead caterpillars (in the fourth-fifth instars) were registered on the tree trunks, with the typical symptoms of *E. maimaiga* infection (Fig. 2C). Mortality of *L. dispar* caterpillars from *E. maimaiga*

Figure 2. Mortality of *Lymantria dispar* caused by: A – *Cotesia melanoscela* (SF Vidin, 19.05.2021); B – *Entomophaga maimaiga* (ME Nessebar, 18.06.2021); C – *Entomophaga maimaiga* (ME Nessebar, 18.06.2021); D – *Entomophaga maimaiga* (SGE Nessebar, 05.07.2021).

was established at the end of June and the beginning of July, in the region of Nessebar. The heavy rainfalls led to the removing of the larvae from the trunks and their accumulation in the base of the trees (Fig. 2D). In the region of Vidin and Balchik, the presence of gypsy moth dead larvae on the tree trunks was registered, but no development of massive epizootic was observed.

Density of the new generation of Lymantria dispar

Only single egg masses of the gypsy moth's new generation were found during the observation in the autumn, in the region of Nessebar. The density was extremely low – 0 to 0.04 egg masses/tree in the forest stands of ME Nessebar (Fig. 3A) and from 0.01 to 0.30 egg masses/tree in SGE Nessebar (Fig. 3B).

In the area of SGE Balchik, the density of the gypsy moth in October, in three sample plots, was lower than in March, and in the other four plots – it was higher (Fig. 3C). The population density of the new generation of the pest (1.4-10.7 egg masses/tree) was preserved approximately at the levels of the previous generation (2.7-15.9 egg masses/tree). In the region of SF Vidin, the population densities of the new generations were significantly lower in four localities, and an insignificant increase in population density in the new generation of the pest was reported in two plots (Fig. 3D).

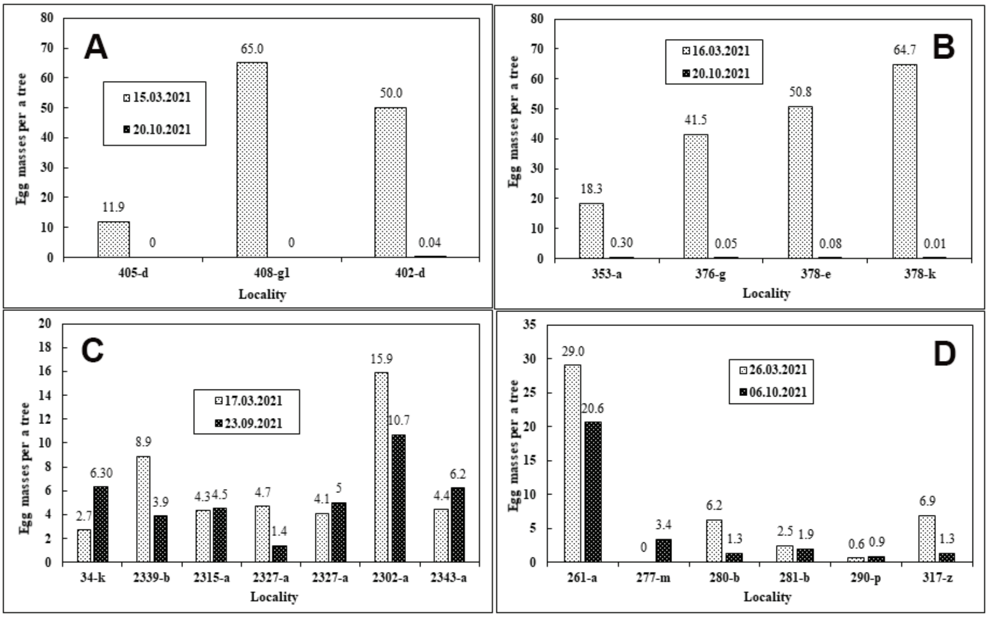

Figure 3. Egg masses of *Lymantria dispar* in 2020 and 2021 populations: A – ME Nessebar; B – SGE Nessebar; C – SGE Balchik; D – SF Vidin.

Discussion

After the introduction in Bulgaria, the fungus *E. maimaiga* has constantly expanded its range in the East European countries. In 2011, the pathogen was registered in two localities of the gypsy moth in Serbia (Tabaković-Tošić et al., 2012) and two localities in the European part of Turkey (Georgiev et al., 2012). In 2012, it was found in one locality in northern Greece and three localities in Northern Macedonia (Georgieva et al., 2013). In the same year, mortality of *L. dispar* was observed with symptoms typical for the *E. maimaiga* infection in three localities in southern Romania (Netoiu et al., 2016). In 2013, *E. maimaiga* was found in nine localities in Croatia (Hrašovec et al., 2013), five localities in Bosnia and Herzegovina (Milotić et al., 2015), seven localities in Hungary (Csóka et al., 2014) and two localities in Slovakia (Zubrik et al., 2014). In the following years, the range of the pathogen expanded in the countries in which it has established its presence (Hrašovec et al., 2013; Netoiu et al., 2016; Zubrik et al., 2016). In 2019, an epizootic of *E. maimaiga* was found in two localities of the gypsy moth in Austria (Hoch et al., 2019) and five localities in the Czech Republic (Holuša et al., 2020).

Site-specific factors (occurrence of rainfalls and high atmospheric humidity in May-June) also affect the prevalence of the fungus *E. maimaiga* and its efficiency to suppress the gypsy moth outbreaks (Georgiev et al., 2013, 2020). In the current experiments, the release of *E. maimaiga* in the region of Nessebar was successful, although the lack of precipitation in the spring did not allow the development of an early epizootic, and as a result, by mid-June, caterpillars of *L. dispar* completely defoliated the infested forest stands. However, heavy rainfalls in June favoured the development of the pathogen, which suppressed the outbreak of the pest. The results in the area of SFE Vidin and Balchik were worse, but the high number of *E. maimaiga* resting spores still remaining in the surface layers of the soil in these areas has the potential to suppress *L. dispar* attacks in years to follow. In this regard, it is appropriate not to conduct additional pest control, but to rely on the pathogen to cause epizootic under more favourable conditions in 2022. Such results were registered in 2012 on the Central Black Sea coast, after the release of *E. maimaiga* in 2011 in the area of SGE Staro Oryahovo (Georgiev et al., 2013).

Until the introduction of *E. maimaiga* in Bulgaria, the attacks of *L. dispar* during its outbreaks amounted to 492-1028 thousand ha, and after the introduction, they decreased to 23-90 thousand ha (Georgiev et al., 2020).

In conclusion, it should be emphasized that the use of *E. maimaiga* for biological control of the gypsy moth populations, under favourable conditions, contributes to reducing costs, preserving biodiversity and improving the recreational qualities of forest ecosystems. However, the effectiveness of the pathogen strongly depends on the specific environmental conditions – that is why it should only be used as a part of an integrated programme to control the pest.

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