

ENTOMOPHAGA MAIMAIGA IN ROMANIA AND FUTURE POSSIBILITIES IN BIOLOGICAL CONTROL OF *LYMANTRIA DISPAR* POPULATIONS

NETOIU CONSTANTIN¹

TOMESCU ROMICA¹

ILIESCU OVIDIU¹

BUZATU ANDREI¹

¹INCDS Marin Dracea

e-mail: c_netoiu@yahoo.com

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ABSTRACT

Once the pathogen *Entomophaga maimaiga* was introduced in Bulgaria (1999), they were reported *Lymantria dispar* larvae high mortality in Romania during 2004-2006 outbreak which developed in wet mixed forests of oak (*Q. petraea*) and beech (*F. sylvatica*) in the South-West of the country in Baile Herculane, Orsova and Modova Noua Forest Districts. Larvae mortality were reported at the beginning of the 2013-2015 *Lymantria dispar* outbreak in several Turkey oak (*Q. cerris*) and Hungarian oak (*Q. frainetto*) forests in Southern Romania (Ghimpati, Slavesti, Costesti) and in the years 2013 and 2014, the pathogen acted over a large area, causing significant mortality. The first field and laboratory experiments regarding the possibilities of biological control of *Lymantria dispar* populations using *Entomophaga maimaiga* were installed in 2014. Using cadavers of *Lymantria dispar* larvae with symptoms of the disease caused by *Entomophaga maimaiga* collected from forests in Costesti area, various experiments were installed in the field, pursuing the possibility of spreading and establishing the pathogen. There have been used a design with two influencing factors: A - the form of the dispersed material (powder - suspension); B - forest soil moisture (wet - dry). The spores were dispersed in the form of powder resulted from the grinding of dead larvae, and also in the form of water suspension, in wet soil and dry soil conditions. In the laboratory, experiments were performed on the persistence of spores in the soil and also in artificial growth on culture media of spores preserved in laboratory conditions. The results of the experiments are presented in the paper.

INTRODUCTION

Entomophaga maimaiga Humber, Shimazu and Soper 1988 is an entomopathogen of *Lymantria dispar* L., 1758, one of the most harmful defoliators of broadleaves in Europe, Asia and North America.

Originally discovered and described from a gypsy moth population in Japan, (Koraima, 1954) where it periodically caused epizootics, *Entomophaga maimaiga* occurs as *Lymantria dispar* pathogen in other parts of Asia Pacific (Nielsen et al., 2005).

The pathogen was introduced in North America in Massachusetts in 1910-1911 (Speare, Colley, 1912) and it was not reported until 1989, when it caused epizootics in seven North –Eastern States (Hajek et al., 1990).

A detailed review about biology, pathology, host specificity and epizootiology of *Entomophaga maimaiga* was published by Hajek (1999).

In 1999 introduction of the pathogen was conducted in Bulgaria in Karlovo region (Pilarska et al., 2000), followed in the same year and in 2000 by another introductions, in other regions of Bulgaria. In 2005 it was reported the first epizootics caused by *Entomophaga maimaiga* in four regions located 30-70 kilometers from the introduction sites, in other regions of Bulgaria (Pilarska et al., 2006). Since 2011 *Entomophagaimaiga* has invaded several *Lymantria dispar* populations in Bulgaria (Georgiev et al., 2011).

In Central Serbia in two localities –Boracki-Gaj and Bogovada in 2011 where gypsy moth outbreaks were predicted, no considerable defoliation was observed, and high mortality of older instar larvae was reported. The mortality was between 85% and 92%, and in bodies of dead larvae it was revealed the presence of *Entomophaga maimaiga* spores. In 2012 a new epizootic caused by *Entomophaga maimaiga* was reported in the region of Avala hill near Belgrade. In 2013 and 2014 the pathogen was detected in over 100 localities in Serbia. The mortality rate was very high – between 86,7% and 98%, *Entomophaga maimaiga* reducing the *Lymantria dispar* population density and preventing severe outbreaks in Serbia (Tabacovic-Tosic, 2012).

Also in 2011 in the European Turkey *Entomophaga maimaiga* was found in two localities, Vize and Yalikoï, (Georgiev et al., 2012).

An epizootic occurred in 2005 in the region of Kremen (Southern Bulgaria), thus it was presumed that the pathogen crossed into Greece, the neighboring country. Microscopic analysis of larvae collected in the field and reared in laboratory revealed the presence of conidia and resting spores of *Entomophaga maimaiga* in Kidaris, Greece (Georgieva et al., 2013).

In 2012 *Entomophaga maimaiga* was established in FYR Macedonia, causing larval mortality between 8,3% and 12,3% (Georgieva et al., 2013).

The Croatian foresters reported in early summer of 2012 a high incidence of dying gypsy moth larvae, in the Eastern part of Croatia (Hrasovek et al., 2013). In 2014 *Entomophaga maimaiga* was confirmed further in the West, in the middle part of Croatia in forests around the city of Sisak.

In Bosnia and Herzegovina the pathogen was reported in the North-East part of the country, in 2013, at five localities near the Croatian sites where the fungus has been detected (Milotic et al., 2015).

High levels of mortality occurred in an outbreak of the gypsy moth population in Georgia in 2005 and molecular analyses of resting spores confirmed that the pathogen was *Entomophaga maimaiga* (Kereselidze. 2011).

Hajek (1999) quotes Walsh (1996) showing that in a collection of *Lymantria dispar* in Romania, entomophthoralean resting spores found in one cadaver of *Lymantria dispar* differed from that of both *Entomophaga aulicae* and *Entomophaga maimaiga*.

In Romania, the first mortality symptoms specific to *Entomophaga maimaiga*, were observed in late summer 2006 by us, in populations of caterpillars of *Lymantria dispar* in the humid mixed thermophilic oak, beech and hornbeam forests South-West of the country (Herculane, Orșova, Berzeasca), when along with NPV, the pathogen stopped the outbreak (figure 1).



Figure 1. *Lymantria dispar* larvae killed by *E. maimaiga* and NPV. Baile Herculane, 2006.

Moreover, according to the field staff in that area, low mortality was observed as early as the late summer of 2005 but went unreported. Unfortunately, due to poor communications, mortality was not reported in 2006 until July, towards the end of the pathogen activity, so no detailed observations were made on *Lymantria dispar* larval mortality (Tomescu, Nețoiu, 2006).

The next generation (2006-2007) of *Lymantria dispar* population entered into latency, and observations were impossible.

After 5 years, in 2011-2012 generation, in Southern Romania *Lymantria dispar* developed a new outbreak in the forests with mezoxerophile oaks, which until 2014 comprised the majority of oak forests in the plains and low hills of South and West Romania (figure 2).

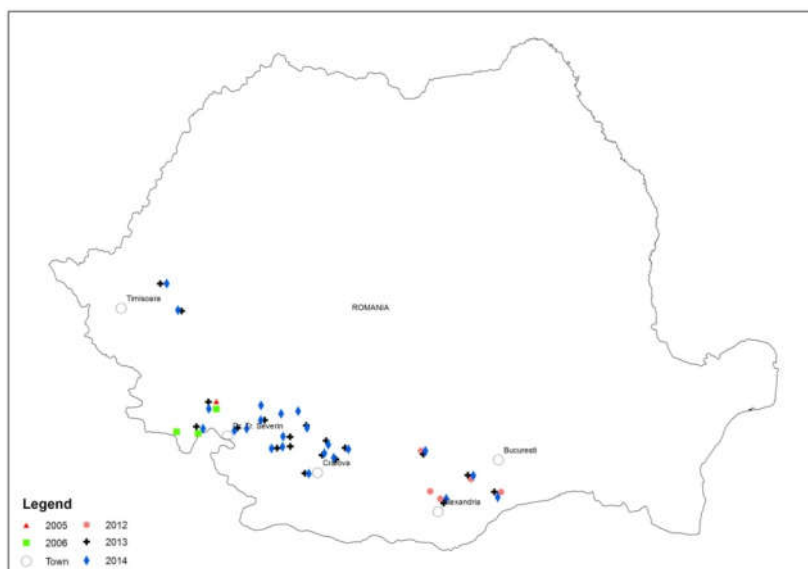


Figure 2. Distribution of *E. maimaiga* in Romania in the last two *L. dispar* outbreaks.

In spring 2012 observations on populations of caterpillars in some of these forests Nebuna (Forest District Ghimpați), Zăvestreni (Forest District Slăvești) and Turcului (Forest District Costești) have revealed the emergence of caterpillars in the four instar with clear signs of *Entomophaga maimaiga* activity, without carrying out laboratory tests to identify the fungus.

In 2012/2013 and 2013/2014 generations, the outbreaks has grown both in surface and in intensity, comprising over 50 thousand hectares.

In spring 2013, under conditions of high infestations were applied aerial treatments with Dimilin SC 48 on an area of 35 thousand hectares, and, amid a very wet spring, among *Lymantria dispar* caterpillars were recorded very high mortality due to the pathogen, which allowed collecting large quantities of cadavers (figure 3).



Figure 3. *L. dispar* larvae dead on tree trunk and collected cadavers, Balcesti, 2013.

In 2014, with a rainy spring and inoculation actions with fungal material collected in 2013, the epizootic manifested itself all over the range of the outbreak, as predicted, and the aerial treatments were cancelled.

MATERIALS AND METHODS

After noting the appearance of symptoms of mortality specific to *Entomophaga maimaiga* among populations of *Lymantria dispar* larvae in 2012 and particularly in 2013 and 2014, when manifested in the form of an epizootic, they were made observations on the evolution of mortality and has been initiated an ample action with the participation of colleagues from Forest Administration for the collection of dehydrated larvae cadavers.

In 2012 (July) were collected from small outbreaks 300 grams of dried cadavers in 3 locations (Nebuna, Costești, Zăvestreni). The material was subjected to natural controlled drying in a cool, dry and well ventilated room. After drying the cadavers were grounded mechanically and stored refrigerated until the spring of 2013.

Because in the next generations were manifested epizootics caused by *Entomophaga maimaiga* over large areas of forests in Southern Romania, collecting cadavers of *Lymantria dispar* grew, resulting in the gathering of about 5 kilograms of dried fungal material, kept also refrigerated. The bulk of this fungal material was used in spring 2014(immediately after the melting of snow in the forest) in the inoculation of the fungus in many other forests infested with *Lymantria dispar*, where the pathogen had not acted. The results of pathogen inoculation in new areas have been encouraging, this being the object of another work. A small part of fungal material was stored refrigerated for future experiments, while the other was used in various experiments placed in the field and in the laboratory during the years 2014 and 2015.

The first experiment was placed in the laboratory with the objective of observing how the azygospores spread in suspension and the number of azygospores in water suspensions with different ratios of dispersion phase and dispersion medium, with the ultimate goal of finding new ways of introducing the pathogen in the forests.

To highlight the presence of azygospores and how the suspension settles, were carried out microscopic analysis (Kruss optical microscope, model MBLKW 2000) on samples taken from aqueous suspension with different rations of the disperse phase (fungal material) and the dispersion medium (water): 1/10, 1/50, 1/100, 1/200.

Counting azygospores per volume unit of the suspension was performed with counting chambers type Burker-Turk on 10 samples from each suspension, the ratio between the two parts of the suspension being 1/200, in various states of sedimentation.

Another experiment was placed in a laboratory, in order to follow the viability of azygospores while maintaining the fungal material refrigerated. Fungal material milled and stored 1,2 and 3 years was inoculated on various culture media, which were then put to incubate in favorable temperature(20⁰C), relative humidity(80-90%), and light(8 hours/day) conditions,in the growth chamber type Binder KBW 240. As culture media were used wet filter paper, egg whites and artificial culture media Sabouraud CAN 1 and Sabouraud CAN 2. Observations were made daily for 7 days, macro and microscopic, following the formation of fungichyphaes (figure 4).

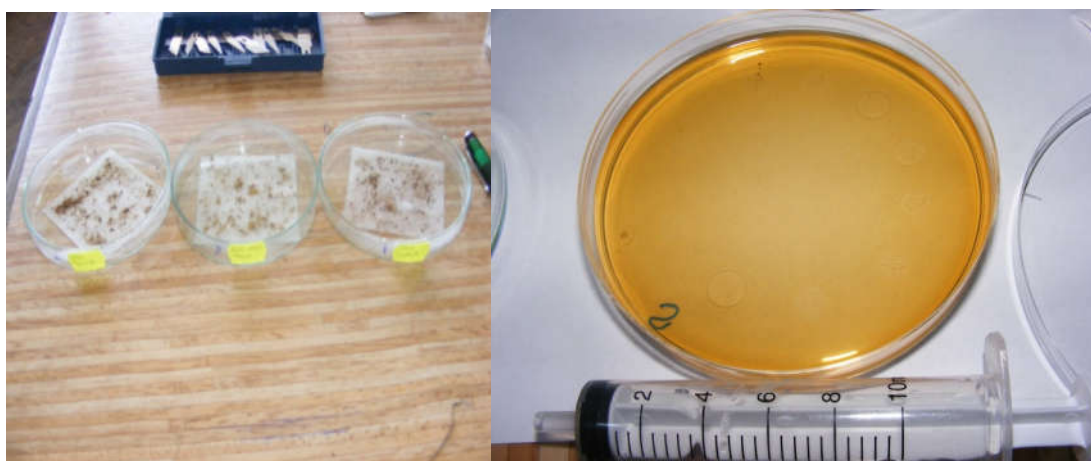


Figure 5.Fungal material inoculation on various culture media.

In order to highlight the spread and the survival time of azygospores in the soil of the forest, in fall 2015 soil samples were collected from an experimental area placed since spring 2013 (Balota Forest), where inoculations had been made with fungal material under different humidity conditions (humid and dry, spraying and dusting), and, in summer 2013 and 2014 occurred mortalities among *Lymantria dispar* larvae. The experiment is ongoing and will be the subject of a new article.

RESULTS AND DISCUSSIONS

Highlighting the presence and number of fungal spores in water suspension

Having the fungal material collected in previous years and kept in powder form refrigerated, by microscopic analysis on samples extracted from the aqueous suspension, it was revealed the presence of spores in all samples analyzed, regardless of the ratio of the dispersed phase and dispersion medium.

Dispersal of spores suspended in water differed greatly with the degree of sedimentation of suspension, which in turn depends on the ratio of the dispersed medium, the greater this ratio, the sedimentation rate is faster.

After testing several suspensions with different phases and reports between the two sides of dispersed systems, 1/200 report was shown to be the best for counting the resistant spores in the Burker-Turk counting chambers (figure 6).

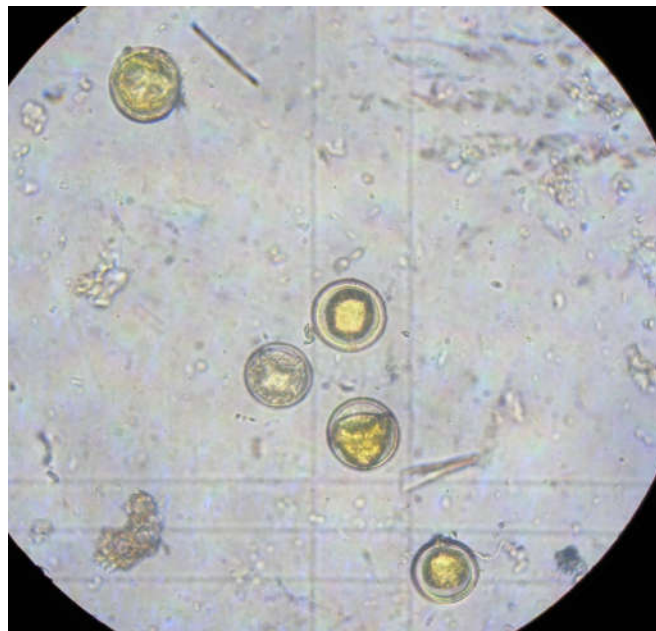


Figure 6. Counting the resistant spores in the Burker-Turk chambers

This suspension has been shown to have a quite intense sedimentation process, so, as to count the average number of azygospores, were analyzed different states by sedimentation: homogenized by manual shaking suspension, suspension free, residue.

The results of the number of spores in suspension varied widely depending on the state of sedimentation (table 1).

Given the great differences between the average number of azygospores per unit volume and the variation coefficients for the types of suspensions analyzed for future inoculations, it is recommended that the suspension to be homogenized by shaking throughout the application.

Variation of resistant spores average number depending on the state of sedimentation

Table 1

States by sedimentation	Number of samples	Resistant spores average number/ml	Standard deviation	Variation coefficients (%)
Homogenized 1/200	10	$1,5 \times 10^4$	0,70	47,14
Suspension free 1/200	10	3×10^3	0,45	152,75
Residue 1/200	10	$14,8 \times 10^4$	2.71	18.33

To establish *Entomophaga maimaiga* in new areas using resistant spores requires a relatively small number of spores (6×10^5 resting spores per 0.01 ha plot) collected from the field (Reardon, Hajek, 1998). At an average number of 1.5×10^4 azygospores per ml, we consider a quantity of 40 ml resistant spore suspension per 0.01 ha plot (4 l/ha), stirred and uniformly distributed, effective to successfully spread the pathogen in the field.

Maintaining the viability of azygospores while held under refrigeration

Macro and microscopic observations performed daily on the evolution of the pathogen in the culture media tested, showed that on filter paper and egg white fungal hyphae were not formed, probably due to lack of specific substances of the fatbody of *Lymantria dispar* larvae.

On the culture media Sabouraud CAN-1 and Sabouraud CAN-2, three days after inoculation, we have seen the development of fungal mycelia with diameters between 6 and 22 mm, higher on Sabouraud CAN-1 culture media (figure 7).

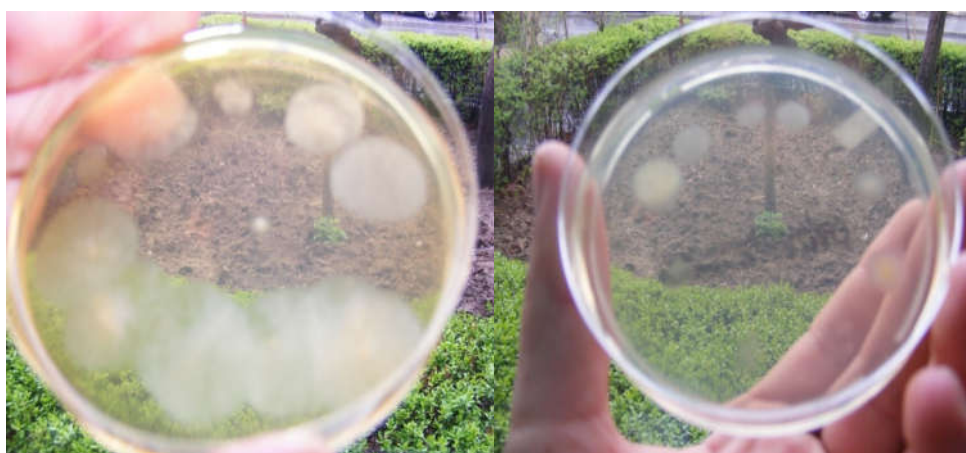


Figure 7. Culture media Sabouraud CAN-1 and Sabouraud CAN-2, three days after inoculation.

After seven days of inoculation on both culture media, the mycelia have a good growth, covering almost all of the entire surface of the culture dishes, and the free space in the dishes was colonized by a dark grey mold species (figure 8).

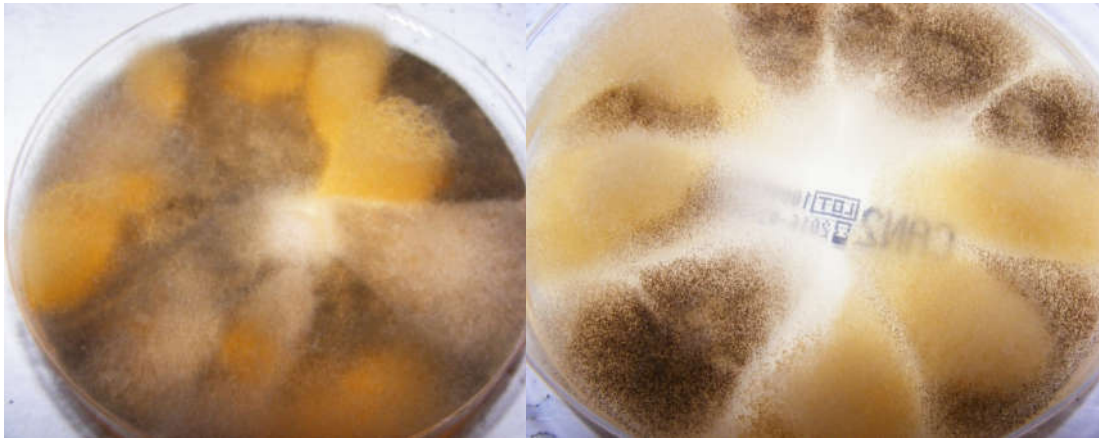


Figure 8. Culture media Sabouraud CAN-1 and Sabouraud CAN-2, seven days after inoculation.

After microscopic analysis of the micelles formed, it was observed abundant presence of conidiophores specific to *Entomophaga maimaiga* (figure 9).

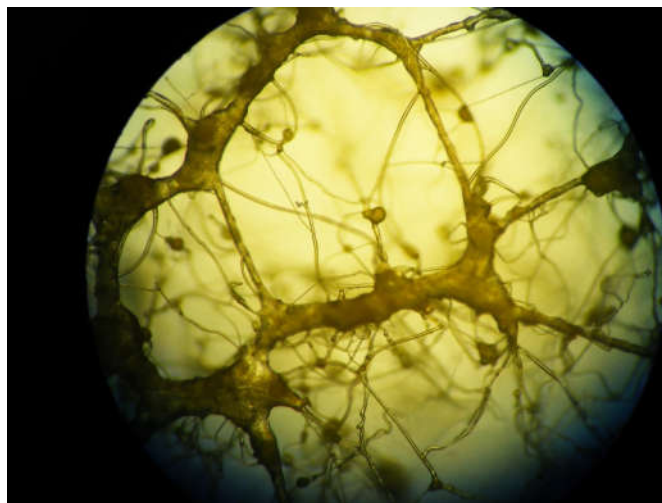


Figure 9. *Entomophaga maimaiga* hyphal bodies.

Conidiophores development on artificial culture media open future possibilities of pathogen multiplication in the laboratory and its subsequent release in nature.

CONCLUSIONS

After the pathogen *Entomophaga maimaiga* was introduced in Bulgaria in 1999, it has rapidly spread throughout the Balkan Peninsula, including Romania, causing significant epizootic, which together with NPV, contributed to halting *Lymantria dispar* outbreaks developed later (2003-2006 and 2012-2015) in the region. Following the epizootic recorded in 2013 and 2014, it was managed to collect a large amount of

cadavers infected with *Entomophaga maimaiga*, which allowed inoculation of the fungus in new areas in Romania, placement of experiments in order to track the spread of the fungus in space and time and find new possibilities of using the pathogen as a mycoinsecticid.

An average number of 1.5×10^4 resistant spores per ml that we have achieved in water suspensions of fungal material dried and finely milled, revealed that an application rate of 40 ml suspension per 0.01 hectares would be sufficient to inoculate the fungus on that surface.

Laboratory tests on the viability of resistant spores in fungal material preserved one, two or three years under refrigeration, showed that spores placed under favorable conditions on culture media (Sabouraud CAN-1 and Sabouraud CAN-2) forms micelya, conidiophores and conidia, specific to *Entomophaga maimaiga*.

The results obtained in the laboratory opens future possibilities for use of the pathogen at a larger scale in the biological control of *Lymantria dispar*.

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