ACTA ZOOLOGICA BULGARICA
Acta zool. bulg., 65 (2), 2013: 173-177

Bioassays for Detection of the Entomopathogenic Fungus *Entomophaga maimaiga* (Entomophtorales: Entomophtoraceae) in Soil From Different Sites in Bulgaria

***Daniela Pilarska*[1,5], *Milcho Todorov*[1], *Plamen Pilarski*[2], *Virginia Djorova*[1], *Leellen Solter*[3], *Georgi Georgiev*[4]**

[1] Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 2 Gagarin St., Sofia 1113, Bulgaria; E-mails: dpilarska@yahoo.com, mtodorov@zoology.bas.bg
[2] Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. Georgi Bonchev St., bl. 21, Sofia 1113 Bulgaria; E-mail: psp@bio.bas.bg
[3] Illinois Natural History Survey, Prairie Research Institute at the University of Illinois, 1816 S. Oak St., Champaign, IL 61820, USA; E-mail: lsolter@illinois.edu
[4] Forest Research Institute, Bulgarian Academy of Sciences, 132, St. Kliment Ohridski Blvd., Sofia 1756, Bulgaria; E-mail: ggeorg@bas.bg
[5] Czech University of Life Sciences, 129 Kamýcká St., 16521 Prague 6, Suchdol, Czech Republic

Abstract: Gypsy moth, *Lymantria dispar,* larvae were exposed to soil extracted from 16 sites in Bulgaria where natural gypsy moth populations occur. Azygospores of *E. maimaiga* were produced in larvae exposed to 11 of the soil samples. Host mortality caused by the fungus varied between 3.3 and 43.3%. The percentage of larvae that died from unknown causes ranged from 3.3 to 66.7%. The results of this study show that the fungal pathogen is widely distributed in Bulgaria and is persisting in sites where epizootics have occurred, a precondition for successful and sustainable control of *Lymantria dispar*.

Key words: *Lymantria dispar*, *Entomophaga maimaiga*, soil samples, bioassays, Bulgaria

Introduction

Entomophaga maimaiga is a naturally occurring obligate fungal pathogen specific to gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) larvae. It was isolated from US gypsy moth populations long after attempts at introduction were made. The US isolates were identified as originating from *L. dispar* in Japan (Hajek 1999). *E. maimaiga* is also a natural enemy of gypsy moth in other parts of Pacific Asia (Nielsen *et al*. 2005)and the pathogen is now well established in North America and is considered the most important natural enemy of this pest insect in the northeastern US (Hajek *et al.* 2004).

E. maimaiga produces two kinds of spores, conidia and azygospores. Conidia are formed externally on early season hosts and serve to spread infection within the spring larval population. Thick-walled azygospores, or resting spores, overwinter in the soil and germinate in the spring to infect a new generation of gypsy moth larvae. Azygospores can persist in soil up to12 years after an epizootic (Hajek *et al.* 2000, Hajek *et al.* 2004), thus assuring long term survival of the pathogen during periods when host is not present or active (Pell *et al.* 2001), and sometimes allowing high levels of infection when density of host populations is low (Hajek *et al.* 1990; Elkinton *et al*. 1991).

The first successful introduction of *E. maimaiga* in Europe was conducted in 1999 in Bulgaria (Pilarska *et al.* 2000). The fungus was imported from the US and was introduced via *L. dispar* ca-

davers containing azygospores. In the period 2000-2011, *E. maimaiga* was additionally introduced in ten different gypsy moth populations in each major geographic section of Bulgaria (Georgiev *et al.* 2011). In 2005, *E. maimaiga* epizootics occurred at four different sites in northwest and south central Bulgaria, located 30-70 km from the first three introduction sites in 1999 and 2000 (Pilarska *et al.* 2006). The fungus was recovered in two more localities in northeast and southeast Bulgaria in 2009 (Georgiev *et al.* 2011), sites where no introduction or previous epizootics had occurred. *E. maimaiga* most likely dispersed to the new sites via windblown conidia, similar to the situation in the US where the fungus spread from 7 northeastern states in 1989 to 12 states in 1992 (Hajek *et al.* 2005). Moreover, Dwyer *et al.* (1998) estimated that *E. maimaiga* spread more than 100 km per year in North America in conditions of a relatively dry spring. To evaluate persistence and spread of *E. maimaiga* in Bulgaria, we used bioassays to detect the presence of *E. maimaiga* in soil samples where gypsy moth populations occur.

Materials and Methods

Experimental larvae

L. dispar larvae were obtained from egg masses provided by the USDA-APHIS-PPQ Laboratory, Buzzards Bay, MA, USA. Larvae were hatched and reared on meridic wheat germ diet (Bell *et al.* 1981) in 250-ml plastic cups at 20° C, 16h light/8 h dark. Early instar larvae were used for the bioassays.

Soil samples

Soil samples were collected during the month of March in 2009, 2010 and 2011 from 16 sites in different geographic areas of Bulgaria, including eight sites where *E. maimaiga* was originally introduced, four sites to which *E. maimaga* had spread and epizootics occurred, three sites in which *E. maimaiga* was detected in larvae but no epizootics were observed, and one site where *E. maimaiga* was not reported (Table 1).

Each location consisted of a 1000 m^2 study plot with *Quercus* spp. the dominant trees. Four oak trees were selected at approximately 15 m from the center of each plot in each 90 º quadrant. Leaf litter was cleared within 10 cm of base of each tree and an approximately 150 g soil sample was collected from the upper 5 cm, including the organic layer. The sampling equipment was washed and sterilized with 95% ethanol following the digging of each sample. Soil samples from one plot were pooled resulting in one soil sample per study site, and were held at 15ºC, 24 hr dark for one month before beginning the bioassays.

Bioassays

Three treatments were conducted in which gypsy moth larvae were exposed to soil samples: 1. sterile humid soil (negative control), consisting of soil sterilized at 180 ºC for 2 hours and dampened with sterile water; 2. sterile soil with *E. maimaga* azygospores (homogenized infected larva) added after sterilization (positive control), and 3. untreated soils from the sample sites using the methodology described by Hajek *et al.* (2004). The control treatments were conducted only in 2009. All bioassays were conducted in the month of May of the same year the samples were obtained in order to the match germination period of azygospores in the field.

Approximately 20-30 g soil from each study site was placed in a 11 x 4.5 cm plastic container and the soil was moistened with distilled water. Ten larvae were added to each of the containers and were maintained at 15 ºC for 3 days without a food source. The larvae were then transferred to 30-ml plastic cups containing diet, one larva per cup, and were monitored daily for 10 days. Larvae that died were placed in a humid growth chamber and held at 20 ºC for 7 days to allow formation of azygospores, then stored at 4º C in a household refrigerator for a month. Each cadaver was dissected individually and examined under light microscopy (magnification 125x) for presence of *E. maimaiga* conidia or azygospores, or other pathogens. Three repetitions were conducted for each soil sample for a total of 30 larvae per location per year.

Results and Discussion

Mortality of *L. dispar* larvae in the sterile soil treatment (negative control) was 23.3% (Table 2). No azygospores or mycelia of *E. maimaiga* or stages of any other pathogens were detected in the negative control larvae. In the treatment with sterile soil containing *E. maimaiga* azygospores (positive control), mortality reached 70.0%, however, azygospores were observed only in 13.3% of larvae.

In 2009, larvae exposed to 1 of the 5 soil samples we tested became infected with *E. maimaiga* (Table 2). Azygospores of the fungus were detected

Table 1. Main characteristics of the sample plots.

Sample plots	Year *E. maimaiga* introduced or epizootics occurred	Year, soil sample collections	No. larvae tested	Altitude (m)	Geographic coordinates
***E. maimaiga* introduction**					
Gorni Domlyan	1999	2010, 2011	60	375	42°33.150'N, 024°54.032'E
Gabrovnitsa	2000	2009, 2010, 2011	90	481	43°05.331'N. 023°27.626'E
Stryama	2005	2010, 2011	60	182	42°13.710'N. 024°51.659'E
Sadievo	2008	2009, 2010, 2011	90	151	42°31.783'N, 026°08.901'E
Slavyanovo	2009	2011	30	345	43°17.090'N, 026°08.834'E
Ruets	2010	2011	30	312	43°12.071'N, 026°37.570'E
Dalgach	2010	2011	30	193	43°12.579'N, 026°42.287'E
Solnik	2011	2011	30	205	43°54.129'N, 027°42.568'E
***E. maimaiga* epizootics (not introduced)**					
Elovitsa	2005	2009, 2011	60	345	43°19.850'N, 023°00.247'E
Skravena	2005	2010, 2011	30	516	42°57.420'N, 023°49.504'E
Spahievo	2005	2009, 2010, 2011	90	451	42°00.978'N, 025°25.566'E
Kremen	2005	2009, 2010, 2011	90	474	41°17.133'N, 025°19.868'E
***E.maimaiga* reported, no epizootics**					
Ravna gora	–	2010	30	336	42°06.541'N, 027°25.100'E
Zvezdets	–	2011	30	336	42°06.541'N, 027°25.100'E
Karlanovo	–	2010, 2011	60	645	41°33.047'N, 023°25.228'E
No reported *E.maimaiga* occurrence					
Polena	–	2011	30	450	41°50.327'N, 023°05.616'E

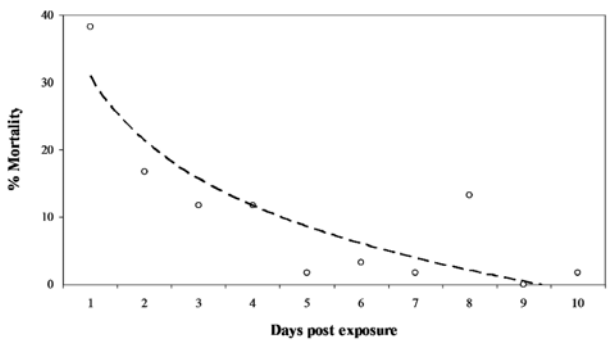

Fig. 1. Mortality of *Lymantria dispar* larvae infected with *Entomophaga maimaga* during a period of 10 days after exposure to soil samples (N=60).

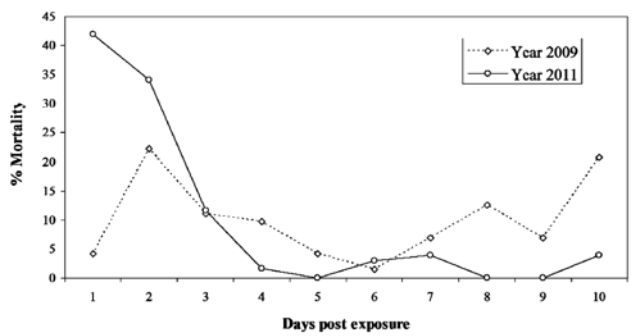

Fig. 2. Mortality due to unknown causes of *Lymantria dispar* larvae exposed to soil samples in 2009 (N=72) and 2011 (N=129).

in 6.7% of larvae exposed to the soil from Kremen.

Infections were recorded in larvae exposed to 7 of the 9 soil samples collected in 2010 (Table 2). Azygospores were not observed in the test larvae exposed to the soil from Stryama, an introduction site, and Spahievo, a site with recorded epizootics. The prevalence of infections in test larvae varied from 3.3% of larvae exposed to the soil from Sadievo to 16.7% of larvae exposed to the soil from Gabrovnitsa and Skravena.

Larvae exposed to 9 of the 16 soil samples in 2011 became infected (Table 2). Azygospores were not detected in the test larvae exposed to the soil from Kremen Gabrovnitsa, Ravna Gora, Slavyanovo, Dalgach, Ruets and Polena. *E. maimaiga* has not been reported in gypsy moth populations in Polena.

The fact that azygospores were not observed each year in some soil samples collected from the same site (e.g. Spahievo, Gabrovnitsa, Stryama, Elovitsa etc.) suggests that either the concentration

Table 2. Mortality of *L. dispar* larvae exposed to soil samples from different sites in Bulgaria during the period 2009-2011.

Soil samples	Year	Mortality [%][c]		
		E. maimaiga	Unknown causes	Total
Negative control [a]	2009	0	23.3	23.3
Positive control [b]	2009	13.3	56.7	70.0
Kremen	2009	6.7	23.3	30.0
	2010	6.7	0	6.7
	2011	0	40.0	40.0
Sadievo	2009	0	30.0	30.0
	2010	3.3	0	3.3
	2011	13.3	16.7	30.0
Gabrovnitsa	2009	0	66.7	66.7
	2010	16.7	10.0	26.7
	2011	0	90.0	90.0
Spahievo	2009	0	0	0
	2010	0	6.7	6.7
	2011	3.3	53.4	56.7
Elovitsa	2009	0	10.0	10.0
	2011	16.7	33.3	50.0
Gorni Domlyan	2010	3.3	6.7	10.0
	2011	10.0	23.3	33.3
Stryama	2010	0	3.3	3.3
	2011	3.3	23.3	26.7
Skravena	2010	16.7	3.3	20.0
	2011	43.3	0	43.3
Karlanovo	2010	6.7	6.7	6.7
	2011	3.3	43.4	46.7
Solnik	2010	3.3	3.4	6.7
	2011	16.6	16.7	33.3
Zvezdets	2011	13.3	30.0	43.3

[a] No *E. maimaiga* spores in sample
[b] *E. maimaiga* azygospores added to sample
[c] Number of larvae per site or control treatment per year = 30

of the spores in the sample year was low or that the spores were dead or dormant. According to Hajek and Humber (1997) and Hajek (1999), under field conditions *E. maimaiga* azygospores typically germinate approximately 9 months after production in the host and ca. 1 to 2 weeks before *L. dispar* eggs begin hatching. Not all azygospores that are present in the soil germinate each year, thus providing a reservoir for the following year (Hajek 2004).

The overall mortality of *L. dispar* larvae caused by *E. maimaga* in 2009-2011 from 1 to 10 days post exposure (dpe) is shown in Fig. 1. The mortality was the highest on 1 dpe, 38.3%, and by 3 dpe 66.7% of all infected larvae died. The last larval deaths were recorded on 10 dpe. Our results correspond to those of Hajek (2004) in which the highest mortality occurred between 1 and 3 dpe when 84.1% of the larvae died.

Larval mortality in the bioassays due to unknown causes is shown in Fig. 2. The results from the 2010 bioassay are not presented graphically because only 11 larvae died from unknown causes. In 2009, larval mortality varied and was the highest on 2 dpe and 8 dpe. However, in 2011, 89.2% of the larvae died by 4 dpe (Fig. 2). No direct causes for mortality of the test larvae were determined and no pathogens were observed in these larvae. It is possible that stress associated with 3 d starvation deleteriously affected the condition of the test larvae. Noting the difference in percent mortality in the positive and negative control treatments, it is also possible that fungal invasion killed the stressed hosts before the

formation of azygospores, suggesting that basing positive findings only on production of azygospores is a conservative evaluation of fungal activity in positive sites.

Viable *E. maimaiga* azygospores were detected in sites with recorded introductions and in sites to which the fungus had spread and epizootics occurred, as well as in sites with no records of epizootics. Our results show that the fungus is spreading from the introduction sites and persisting in invaded sites. Future studies should determine whether *E. maimaiga* has a dampening effect on the outbreaks of these European *L. dispar* populations.

Acknowledgments: This research was supported by the National Science Fund of Bulgaria, Project DO-02-282/2008 and Project DO-02-251/2008. We are grateful to Dr. Danail Takov and Mrs. Elena Tsvetanska for their contributions to this work.

References

Bell R.A., C. D. Owens, M. Shapiro, J. R. Tardiff. 1981. Mass rearing and virus production. – In: Doane, C. C., M. L. McManus (Eds.): The Gypsy Moth: Research toward Integrated Pest Management, U.S. Dept. Agric. For. Serv. Tech. Bull., vol. 1584. USDA, Washington, 599-600.

Dwyer G., J. Dushoff, J. S. Elkington, S. A. Levin. 2000. Pathogendriven outbreaks in forest defoliators revisited: building models from experimental data. – *American Naturalist*, **156**: 105–120.

Elkinton, J. S., Hajek, A. E., Boettner, G. H., Simons, E. E. 1991. Distribution and apparent spread of *Entomophaga maimaiga* (Zygomycetes: Entomophthorales) in gypsy moth (Lepidoptera: Lymantriidae) populations in North America. – *Enviromental Entomology*, **20**: 1601-1605.

Georgiev G., P. Mirchev, B. Rossnev, P. Petkov, M. Georgieva, M. Matova, S. Kitanova, D. Pilarska, P. Pilarski, V. Golemansky, M. Todorov, Z. Hubenov, D. Takov. 2011. Introduction of *Entomophaga maimaiga* and control of *Lymantria dispar* calamities in Bulgaria. – In: Kitanova S. (Ed.): Proceedings of Scientific Conference 'Sustainable management of oak forests in Bulgaria', Primorsko, 72-79. (In Bulgarian, English summary).

Hajek A. E. 1999. Pathology and Epizootiology of *Entomophaga maimaiga* Infectoins in Forest Lepidoptera. – *Microbiology and Molecular Biology Reviews*, 814-835.

Hajek A. E., R. A. Humber. 1997. Formation and germination of *Entomophaga maimaiga* azygospores. – *Canadian Journal of Botany*, **75**: 1739-1745.

Hajek A. E., R. A. Humber, J. S. Elkinton, B. May, S. R. A. Walsh, J. C. Silver, 1990. Allozyme and RFLP analyses confirmm *Entomophaga maimaiga* responsible for 1989 epizootics in North American gypsy moth populations. – *Proceedings of the National Academy of Sciences*, **87**: 6979-6982.

Hajek A. E., M. Shimazu, B. Knoblauch. 2000. Isolating a species of Entomophthorales using resting spore-bearing soil. – *Journal of Invertebrate Pathology*, **75**: 298-300.

Hajek A. E., M. Wheeler. 2004. Using bioassays to estimate abundance of *Entomophaga maimaiga* resting spores in soil. – *Journal of Invertebrate Pathology*, **86**: 61-64.

Hajek A. E., M. McManus, I. Jr. Delalibera. 2005. Catalogue of introductions of pathogens and nematodes for classical biological control of insects and mites. FHTET, USDA Forest Service, Morgantown, 59 p.

Kereselidze M., D. Pilarska, A. Hajek, A. B. Jensen, A. Linde. 2011. First record of *Entomophaga maimaiga* Humber, Shimazu & Soper (Entomophthorales: Entomophthoraceae) in Georgia. – *Biocontrol Science and Technology*, **21**: 1375-1380.

Nielsen, C., Milgroom, M. G., Hajek, A. E. 2005. Genetic diversity in the gypsy moth fungal pathogen *Entomophaga maimaiga* from founder populations in North America and source populations in Asia. – *Mycological Research* **109**: 941-950.

Pell J. K., J. Eilenberg, A. E. Hajek, D. C. Steinkraus. 2001. Biology, ecology and pest management potential of Entomophthorales. – In: Butt T. M., C. W. Jackson, N. Magan. (Eds.): Fungi as Biocontrol Agents: Progress, Problems and Potential. CABI Publications, Wallingford, Oxon, UK, 71-153.

Pilarska D., M. McManus, A. Hajek, F. Herard, F. Vega, P. Pilarski, G. Markova. 2000. Introduction of the entomopathogenic fungus *Entomophaga maimaiga* Hum., Shim. & Sop. (Zygomycetes:Entomophtorales) to a *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) population in Bulgaria. – *Journal of Pest Science*, **73**: 125-126.

Pilarska D., M. McManus, P. Pilarski, G. Georgiev, P. Mirchev, A. Linde. 2006. Monitoring the establishment and prevalence of the fungal entomopathogen Entomophaga maimaiga in two *Lymantria dispar* L. populations in Bulgaria. – *Journal of Pest Science*, **79**: 63-67.

Received: 14.05.2012
Accepted: 06.12.2012