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## POTENTIAL IMPACT OF *ENTOMOPHAGA MAIMAIGA* HUMBER, SHIMAZU, AND SOPER (ENTOMOPHTHORALES ENTOMOPHTHORACEAE) ON THE LEPIDOPTERAN FAUNA INHABITING CORK FORESTS IN SARDINIA (ITALY) <sup>(1)</sup>

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Contarini M., Ruiu L., Pilarska D., Luciano P. – Potential impact of *Entomophaga maimaiga* Humber, Shimazu, and Soper (Entomophthorales Entomophthoraceae) on the lepidopteran fauna inhabiting cork forests in Sardinia (Italy).

Periodic outbreaks of forest defoliators like the gypsy moth cause severe impact to the forest ecosystem, which is normally counterbalanced by the action of their natural enemies, including predators, parasitoids, and entomopathogens. Among the latter, the host-specific fungus *Entomophaga maimaiga* can be very effective under favourable conditions. Whilst its close evolutionary relationship with gypsy moth, this entomopathogen has never been detected in certain forest areas where *L. dispar* is a common pest. The results of three years laboratory assays with two different strains of *E. maimaiga* from Bulgaria and Croatia against Lepidopteran species inhabiting cork oak forests in Sardinia are reported. Significant toxicity and virulence against gypsy moth larvae exposed to soil contaminated with resting spores of the fungus was detected for both strains, even if the strain from Bulgaria was significantly more effective. Significant lethal effects were observed also on *M. neustria* larvae, but a successful development and reproduction of the fungus within insect cadavers was detected only in the gypsy moth. No significant effects were observed on other Lepidopteran species.

Given a proper choice of candidate strains, the introduction of *E. maimaiga* in Sardinia, to manage the disruptive action of the gypsy moth would be desirable.

KEY WORDS: *Lymantria dispar*, *Malacosoma neustria*, entomopathogenic fungi, host specificity, bioinsecticide.

### INTRODUCTION

Different defoliator species are responsible for significant damages to cork oak forests in the Mediterranean environment. Among these, preeminent is the role of moth species with higher reproductive potential, such as the gypsy moth *Lymantria dispar* (L.) (Lepidoptera: Erebidae) and the tent caterpillar *Malacosoma neustria* (L.) (Lepidoptera: Lasiocampidae). Periodic outbreaks of these pests cause serious impact to the forest ecosystem, which is normally counterbalanced by the action of their natural enemies, including predators, parasitoids, and entomopathogens (ALALOUNI *et al.*, 2013).

Among the latter, in the case of gypsy moth, a major role in controlling population density in certain geographical areas is played by the nuclear polyhedrosis virus (i.e. NVP) (DWYER *et al.*, 2000), the microsporidia *Nosema* spp. (SOLTER *et al.*, 2010), and the host-specific fungus *Entomophaga maimaiga* Humber, Shimazu, and Soper (Entomophthorales: Entomophthoraceae) (SIEGERT, 2012). However, due to biological and practical limitations of these microbial agents, the entomopathogenic bacterium *Bacillus thuringiensis* Berliner serovar *kurstaki*

(*Btk*) has so far represented the sole concretely available alternative to contain defoliator infestations over most forest areas (RUIU *et al.*, 2013).

*E. maimaiga* is an entomophthoralean pathogen infectious to larvae of the gypsy moth. Initial host infections are determined by the action of two spore forms: conidia, produced on the outer surface of insect cadavers, and resting spores (azygospores), produced inside dead insects. Under favourable environmental conditions, this entomopathogen can be very efficient in containing *L. dispar* populations (REILLY *et al.*, 2014). Whilst its close evolutionary relationship with gypsy moth is reported (HAJEK, 1999), it still has never been detected in certain forest areas where *L. dispar* is a common pest. This is the case of Sardinia, an island located in the Mediterranean basin, where periodic gypsy moth defoliations cause a severe loss of forest tree leaves in thousands of hectares (CONTARINI *et al.*, 2014). On the other side, this fungal entomopathogen was successfully introduced to Bulgaria from the USA (PILARSKA *et al.*, 2000) and is now rapidly spreading throughout Eastern Europe and neighbouring countries (KERESELIDZE *et al.*, 2011; GEORGIEV *et al.*, 2012; TABAKOVIĆ-TOŠIĆ *et al.*, 2012; GEORGIEVA *et al.*, 2013; HRASOVEC *et al.*, 2013; CSÓKA *et al.*, 2014; ZÚBRIK *et al.*, 2014; MILOTI *et al.*, 2015).

However, the knowledge on *E. maimaiga* is still limited and further studies are needed to gather more information on its biology and insecticidal properties. Although beneficial effects following the introduction of such

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species in local ecosystems are expected, a preliminary evaluation of its possible impact on other entomofauna would be desirable.

The results of three years laboratory assays with two different strains of *E. maimaiga* from Bulgaria and Croatia against *L. dispar* and other Lepidopteran species inhabiting cork forests in Sardinia are reported. The aim of the present study was to evaluate the potential of this exotic fungal entomopathogen as a biological control agent for the gypsy moth and to investigate its possible side effects against other forest moths.

## MATERIALS AND METHODS

### ORIGIN OF ENTOMOPHAGA MAIMAIGA STRAINS AND OF LEPIDOPTERAN SPECIES

Two isolates of *E. maimaiga*, provided by Prof. Daniela Pilarska (Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia, Bulgaria), and Prof. Boris Hrasovec (Faculty of Forestry, University of Zagreb, Croatia) were used in this study. The first was collected in Bulgaria in 2012 and the second in Croatia during an epizootic in 2013 (HRAŠOVEC *et al.*, 2013). In line with regulations for the introduction of non-native microorganisms for use in the laboratory in Italy, specific authorization was obtained from the Italian Ministry of agriculture and forestry (COSVIR 11, Prot. N. 0007590, 03/04/2012). Resting spore suspensions of the fungus were obtained through *L. dispar* infected larvae homogenization.

Native lepidopteran species were collected in cork oak forests in Sardinia, where *Quercus suber* L. represents the prevalent tree species.

During winter (2012-2014), gypsy moth egg masses were collected in a cork oak forest nearby Ploaghe-Chiaromonti (Northern Sardinia, Italy) and incubated at 4 °C. In April, eggs were cleaned and surface sterilized with sodium hypochlorite (2 %) and Tween 80 (1 %) before being incubated at 23 °C in a photoperiod of L16:D8 to favor larval eclosion. Neonate larvae were reared on high-wheat germ diet (BELL *et al.*, 1981) up to the fourth instar (BRODERICK *et al.*, 2000).

Larvae of the tent caterpillar *Malacosoma neustria* (Lepidoptera: Lasiocampidae) were collected in spring from infested oak trees in the same forest areas, while the following lepidopteran species were collected from cork oak forests in the centre of Sardinia: *Catocala nymphagoga* (Esper) (Lepidoptera: Erebidae), *Dryobotodes eremita* (Fabricius) and *D. monocroma* (Esper) (Lepidoptera: Noctuidae), *Dryobota labecula* (Esper) (Lepidoptera: Noctuidae), *Nymphalis polychloros* (L.) (Lepidoptera: Nymphalidae), *Orthosia cruda* Denis & Schiffermuller (Lepidoptera: Noctuidae), *Xanthia (Spudaea) ruticilla* (Esper) (Lepidoptera: Noctuidae).

### LABORATORY BIOASSAYS

Laboratory experiments with different insect species were conducted employing a soil bioassay method involving temporary moth exposition to soil contaminated with *E. maimaiga* resting spores (HAJEK & WHEELER, 2004). For this purpose, fourth instar larvae were starved for 3 days at 15 °C inside plastic boxes (4.5 x 11.0 x 5.0 cm) containing sterilized soil (26 g) incorporated with *E. maimaiga* resting spores (0.8 x 10<sup>3</sup> / g). Separate controls were run using sterilized soil with no fungal spores.

The experimental design involved at least three (up to

five) replications of 10-larvae groups for each treatment and for controls.

Treated and control larvae were then maintained individually at 20 °C and fed on the previously described artificial diet, in the case of *L. dispar*, or fresh *Q. suber* leaves for other moth species. Larval mortality was assessed daily during the following 10 days. Dead larvae were incubated for 24 hours individually in humid growth chamber and inspected during the subsequent 10 days to detect any possible fungal sporulation. The production of fungal conidia on the larvae or resting spores inside infected larval bodies, which represents the fungal reproduction success, was verified under phase microscopy after insect dissection. The presence of other microbials, especially fungi and bacteria, was also assessed.

Toxicity (percentage morality), virulence (time to death), and fitness (reproduction success rate) of the fungus were evaluated in 2012-2013 for *E. maimaiga* strain from Bulgaria and in 2014 for strain from Croatia. These assays were conducted all years with *L. dispar* and *M. neustria*, while in the case of other species, assays were limited to specific years, in relation to the availability of sufficient amounts of larval specimens.

### STATISTICAL ANALYSIS

Statistical analysis were conducted using SAS software (version 9.1) with significance level set at  $\alpha = 0.05$  (SAS INSTITUTE, 2004). Mortality data were submitted to analysis of variance (two factor design: insect species and fungal strain) and treatment means were separated from the controls using Dunnett's test (DUNNET, 1955).

Data on time to death and fungal reproduction rate were analyzed by the General Linear Model (GLM) of ANOVA (two factor design: insect species and fungal strain) and means were separated by least squares means comparison.

## RESULTS

The mortalities of different moth species exposed to soil contaminated with *E. maimaiga* resting spores are shown in Table 1. Significant differences among species were basically due to the considerable morality levels recorded on *L. dispar* larvae exposed to strains from Bulgaria (69.6 %) and from Croatia (20.9 %) in comparison with controls ( $F_{5,28} = 17.08, P < 0.0001$ ). Besides, a significantly high mortality of *M. neustria* larvae involved in bioassays (83.2 %) was recorded. However, this species did not show specific susceptibility to the fungus in line with the results of other moth species. In the case of *L. dispar* significantly different pathogenicity was associated to diverse fungal strains ( $F_{1,28} = 25.89, P < 0.0001$ ).

The average time to death of larvae exposed to both *E. maimaiga* strains was 5.3 days for *L. dispar* and ranged from 3.1 to 4.6 days for *M. neustria* (Table 2), with significant differences in the number of days to death among the two insect species ( $F_{1,221} = 28.59, P < 0.0001$ ) and the two fungal strains ( $F_{1,221} = 6.44, P = 0.0019$ ).

As a result of dead larvae inspection by dissection and microscopy detection of *E. maimaiga* conidia and resting spores, both fungal strains proved to be able to successfully reproduce on *L. dispar*, showing a reproduction rate ( $m \pm se$ ) of  $5.4 \pm 2.92$  % in the case of strain from Bulgaria and of  $12.5 \pm 7.21$  % in the case of strain from Croatia. However, no significant differences between the two fungal strains were observed ( $F_{1,12} = 2.59$ ,

Table 1 – Mortality percentages (Abbott corrected) of Lepidopteran larvae exposed to resting spores of strains from Bulgaria or from Croatia of *Entomophaga maimaiga*

Species	Bulgarian strain	Croatian strain
<i>Lymantria dispar</i>	69.6**	20.9*
<i>Malacosoma neustria</i>	83.2**	3.7
<i>Catocala nymphagoga</i>	3.03	-
<i>Dryobotodes eremita</i>	0	-
<i>Dryobotodes monocroma</i>	25.0	-
<i>Dryobota labecula</i>	37.5	-
<i>Nymphalis polychloros</i>	15.2	-
<i>Orthosia cruda</i>	-	0
<i>Spudaea ruticilla</i>	-	25.0

\*indicate significant differences compared to the control (Dunnet's test: \*\*\*P<0.001; \*\*P<0.01; \*P<0.05).

Table 2 – Time to death (m ± SE) of *Lymantria dispar* and *Malacosoma neustria* larvae exposed to Bulgarian or Croatian strains of *Entomophaga maimaiga*

Species	Bulgarian strain		Croatian strain	
	n <sup>a</sup>	days <sup>b</sup>	n	days
<i>Lymantria dispar</i>	53	5.3 ± 0.22a	44	5.3 ± 0.23a
<i>Malacosoma neustria</i>	52	4.6 ± 0.21a	40	3.1 ± 0.19b

<sup>a</sup>Number of larvae used to calculate the average time to death.

<sup>b</sup>Means followed by diverse letters are significantly different (GLM of ANOVA, followed by LSD test: P<0.05).

P = 0.1163). No evidence of successful reproduction of this entomopathogen on *M. neustria* larvae was observed.

The proportion of major microbial groups (*E. maimaiga*, other fungi, bacteria) associated to gypsy moth dead larvae and other causes of death (others) are shown in Fig. I. As detected by microscopy observations, the number of dead larvae containing *E. maimaiga* resting spores in their body was lower compared to other unidentified fungi or bacteria, probably representing secondary pathogens ( $F_{3,24} = 11.09$ ,  $P < 0.0001$ ). Occasional was the detection of a microsporidium (*Nosema* sp.). A significant percentage of larvae did not show any detectable septicaemia, thus supporting a possible direct toxicity and lethal action of *E. maimaiga* against a large proportion of treated insects. On the other side, no significant differences between the two *E. maimaiga* strains in the proportion of major microbial groups and “others” was observed ( $F_{1,24} = 0.00$ ,  $P < 0.9994$ ).

## DISCUSSION AND CONCLUSION

*Entomophaga maimaiga* strains isolated during recent epizootics in Bulgaria and Croatia showed significant toxicity and virulence against gypsy moth larvae collected in cork oak forests in Sardinia, where this fungal pathogen has never been detected (CONTARINI *et al.*, 2013). While the potential of the strain from Croatia against the same Sardinian population has recently been highlighted (CONTARINI *et al.*, 2015), we report for the first time a

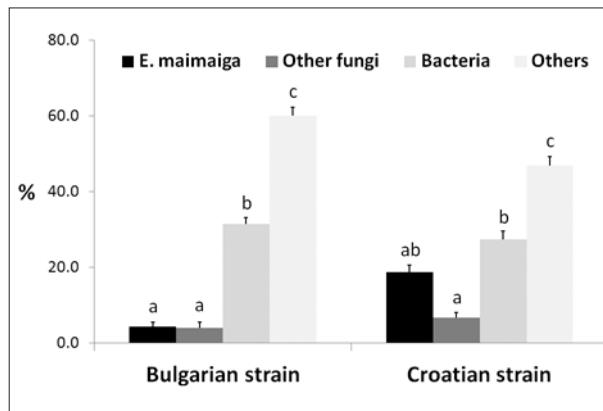


Fig. I – Successful reproduction rate (m ± SE) of two *Entomophaga maimaiga* strains on *Lymantria dispar* and proportion of other microbial groups associated to dead gypsy moths.

\*Different letters above columns indicate significant differences among means (GLM of ANOVA, followed by LSD test: P<0.05).

higher susceptibility of *L. dispar* larvae from the same area to the fungal isolate from Bulgaria. This strain proved to be more effective in terms of achieved larval mortality in comparison to the strain from Croatia. This finding and the possible difference between these strains are in line with the independent recovery of *E. maimaiga* in Croatia (HRAŠOVEC *et al.*, 2013) more than ten years after it was introduced to Bulgaria from the USA (PILARSKA *et al.*, 2000). In general, a variable susceptibility of *L. dispar* to *E. maimaiga* strains of diverse origin can be expected (NIELSEN *et al.*, 2005), as normally occurring for a variety of entomopathogens and their hosts (BOUCIAS & PENDLAND, 1998; RUIU, 2015). Despite a significantly higher susceptibility of gypsy moth larvae to the fungal isolate from Bulgaria in terms of larval mortality, non significant differences among the two isolates in the time to death (virulence) were observed. In addition, both strains were able to successfully develop and reproduce within *L. dispar* larvae and no significant differences in the reproduction rate, comparing the two strains, were noticed. In the case of both strains, the proportion of dead larvae bearing *E. maimaiga* resting spores and those showing different signs and symptoms after death are in agreement with a direct toxic action of the germinating fungus (HAJEK *et al.*, 1996).

An important mortality level of *M. neustria* larvae exposed to soil contaminated with resting spores of the *E. maimaiga* isolate from Bulgaria was also assessed, while non significant were the lethal effects caused by the isolate from Croatia in comparison to the control. On the other hand, there was no evidence of successful *E. maimaiga* reproduction inside larval bodies, which would support the possible co-involvement of other causes of death like other microorganisms (i.e. bacteria, virus, fungi) larvae came into contact with, before achieving the fourth instar and being field collected for our laboratory bioassays.

Whilst virulence features of *E. maimaiga* acting against insects are still a matter of study, it is known that the production of a variety of specific toxins, metabolites, and enzymes (i.e. chitinases, proteases), represent a main trait of entomopathogenic fungi (CASTRILLO *et al.*, 2008; VEY *et al.*, 2001). *E. maimaiga* strains could act in a similar fashion and this might explain some of the differences observed on the two isolates from the Balkan Peninsula. It

would be very important to analyse different strains from that area in a broader comparison so as to identify the fungal strains with the highest potential against diverse European populations of gypsy moth. This approach is particularly needed in areas where the entomopathogen could be successfully introduced.

Another aspect to be considered in order to evaluate the environmental consequences following the introduction of an exotic organism is the possible impact on non-target species (LYNCH *et al.*, 2002). Remarkably, in the case of various Lepidopteran species other than *L. dispar*, neither significant lethal effects nor the ability of the fungus to reproduce within their body were noticed.

For all these reasons, the introduction of *E. maimaiga* in different areas of Italy, including Sardinia, to counterbalance the disruptive action of the gypsy moth would be desirable. On the other side, future studies on *E. maimaiga* will clarify the molecular mechanisms sustaining the insecticidal action, which may lead to the identification of isolates with increased biological properties, thus broadening the choice of candidate strains.

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#### RIASSUNTO

#### POTENZIALE IMPATTO DI ENTOMOPHAGA MAIMAIGA HUMBER, SHIMAZU, AND SOPER (ENTOMOPHTHORALES: ENTOMOPHTHORACEAE) SULLA LEPIDOTTEROFAUNA DELLE FORESTE DI QUERCIA DA SUGHERO IN SARDEGNA (ITALIA).

Le periodiche gradazioni di defogliatori forestali come *Lymantria dispar* sono causa di severe ripercussioni sull'ecosistema foresta, il che stimola anche l'azione di nemici naturali come predatori, parassitoidi ed entomopatogeni. Tra gli ultimi, l'efficacia del fungo entomopatogeno specie-specifico *Entomophaga maimaiga* può essere molto significativa se le condizioni sono favorevoli. Nonostante le strette relazioni evolutive tra i due, questa specie fungina non è mai stata rinvenuta in aree in cui il lepidottero rappresenta una specie ben radicata. Nel presente lavoro si riportano i risultati di tre anni di sperimentazioni in laboratorio con due differenti ceppi di *E. maimaiga* isolati in Bulgaria e in Croazia, e saggiate su larve di differenti specie di lepidotteri comuni nella sugherete della Sardegna. Una significativa tossicità e virulenza è stata osservata su larve di *L. dispar* mantenute su suolo contaminato con azigospore del fungo, evidenziando una maggiore efficacia del ceppo bulgaro rispetto a quello croato, che è comunque risultato efficace. Significativi effetti letali sono stati osservati anche su *Malacosoma neustria*, ma non sugli altri lepidotteri impiegati nei biosaggi. Ad ogni

modo, lo sviluppo e la riproduzione del fungo nel corpo d'insetti infetti sono stati confermati solo nel caso di *L. dispar*. Dopo un'adeguata scelta dei ceppi più attivi, l'introduzione di *E. maimaiga* in Sardegna per il biocontrollo dei principali lepidotteri defogliatori, potrebbe essere vantaggiosa.

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