Infectivity of Hibernated Resting Spores of *Entomophaga maimaiga*
HUMBER, SHIMAZU et SOPER (Entomophthorales:
Entomophthoraceae)

Mitsuaki SHIMAZU, Chikara KOIZUMI, Tamotsu KUSHIDA
and Jun MITSUHASHI

*Forestry and Forest Products Research Institute, P.O. Box 16,
Tsukuba Norin Kenkyu Danchi-nai, Ibaraki 305, Japan*

(Received November 29, 1986)

Infectious activity of hibernated resting spores of *Entomophaga maimaiga* in the field was bioassayed on gypsy moth larvae at the site where an epizootic had occurred the previous year. Mortality of the larvae with this fungus was 7.5 to 14.0% when they were exposed to the cadavers on a tree trunk for 1 night. Suspensions of the cadavers, litter, and soil, which were collected from the field and were thought to contain resting spores, had infectious activity to the larvae, i.e., 4.0 to 12.0% of them were infected with the fungus. When the larvae contacted those materials during rearing, 8.0 to 44.0% of them were also infected. The larvae were not infected in the experiments with resting spores 2 years old. No larvae in the control plot were infected in any experiment. From those results, it was ascertained that the infection of young larvae with overwintered resting spores initiated the epizootic in the population of gypsy moth with *E. maimaiga*.

INTRODUCTION

At the terminal stage of an outbreak of gypsy moth, *Lymantria dispar*, epizootics of *Entomophaga maimaiga* HUMBER, SHIMAZU et SOPER often occur in its population in Japan. Sometimes the population is completely destroyed by this fungus, and the fungus has thus been thought one of the most important natural control agents against gypsy moth in Japan (KOYAMA, 1954).

In general, entomophthoraceous fungi produce 2 different types of spores, conidia and resting spores. Resting spores are thought to be a stage tolerant to severe climatic conditions such as winter, while conidia are a stage of dispersion. Little is known of the life cycles of entomophthorales. In the case of *E. maimaiga*, it has been clarified that its transmission from one larva to another is through its conidia (SHIMAZU and SOPER, 1986). However, the mechanism for the initiation of an epizootic of this fungus in a gypsy moth larval population is still unknown. AOKI (1980) reported that the fungus hibernates in the stage of resting spore and that some resting spores germinate in spring. In order to determine its life cycle in the field and to confirm that hibernated resting spores of this fungus are the source of infection of the larvae in spring, we examined by bioassay the infectivity of resting spores which remained at the site of an epizootic which had occurred the previous year.

MATERIALS AND METHODS

Experimental field. The site selected in this study was a forest of *Lithocarpus aedulis* (Fagales: Fagaceae) located at Kominato, Chiba Prefecture, where an outbreak of gypsy moth occurred in 1984; an epizootic of *E. maimaiga* was prevalent in its larval population. The larvae killed by this fungus had attached themselves to the tree trunks, and many of these cadavers still remained in April of 1985 (Fig. 1).

Examination of infectivity. The inside of the cadavers was filled with resting spores. We examined the infectivity of the resting spores by putting the laboratory reared gypsy moth larvae in contact with them in the following manner.

1. *Exposure of larvae to the cadavers on the trunk.* The larvae were released on tree trunks on which cadavers remained. The trunk was wrapped with a sheet of cheesecloth (Kuremona®) 50 × 100 cm, both edges of the sheet were stapled together to make a sleeve and both ends of the sleeve were closed with string. Fourth instar larvae of gypsy moth which had been hatched and reared in our laboratory were released in these sleeve cages, 25, 50, or 100 larvae into each cage. The releasing was done on April 25, 1985. The larvae were collected after 20 hr, and were reared in plastic cups in the laboratory at room temperature, fed on the leaves of birch, *Betula platyphylla*. A similar experiment was also conducted on May 21, 1986, but 25 larvae of 5th instar were released in each cage, and were fed on the leaves of larch, *Larix leptolepis* after re-collection.

2. *Application of the suspension of materials collected in the field to larvae reared in the laboratory.* The following materials were collected on the day of caging experiments: the cadavers on the tree trunk, litter and soil under the tree on which the cadavers

Fig. 1. Cadavers of gypsy moth larvae remaining on the tree trunk of *Lithocarpus aedulis*. (Courtesy of K. KANAMITSU.)

remained. Five days after the collection of these materials, 0.8 g of cadavers, 10 g of litter, and 20 g of soil were suspended in 100 ml of water, and filtered with gauze. The laboratory-reared 4th instar larvae were dipped into these filtrates for 5 sec, air dried, and reared on birch leaves in plastic cups. Other 4th instar larvae were forced to feed on the birch leaves dipped in the same filtrates and air dried. These experiments were conducted in May of 1985.

3. *Rearing of larvae with the materials collected in the field.* Part of the above mentioned materials collected in the field was stored in a refrigerator. After 1 month, these materials were placed in plastic cups. Laboratory-reared 2nd instar larvae were confined in these cups and were exposed to the materials. The larvae were kept with the cadavers for 2 hr, 24 hr, and the entire rearing period, and with litter and soil for the entire rearing period. Birch leaves were supplied to the larvae. A similar experiment was also conducted in 1986. Materials were collected on May 21, 1986 and given to the laboratory-reared 4th instar larvae for the entire rearing period; larch leaves were supplied to the larvae.

In all the experiments, insect mortality was checked daily. A dead larva was taken out of the rearing cup, placed in a petri dish with moistened filter paper, and the cause of death was examined with a microscope.

RESULTS AND DISCUSSION

Some of the larvae released on a tree trunk on which cadavers remained were infected with *E. maimaiga* in all the released plots (Table 1). Percentage of those infected was 7.5 to 14.0%, and the periods required for death were around 15 days. This incubation period was longer than that caused by conidial infection (SHIMAZU and SOPER, 1986) at about the same temperature of 20 to 25°C. No effects of larval density on infection were observed.

Infection by *E. maimaiga* occurred in the larvae in all the suspension-treated plots, except the plot containing leaves treated with soil suspension (Table 2). Infection occurred even in the larvae which fed on leaves dipped in a suspension of cadavers or litter. However, it was not certain whether the larvae were infected with the fungus through their digestive systems or not. Since entomophthoraceous fungi usually infect insects percutaneously, it is possible in this case that the larvae were infected with the fungus by germ conidia from the resting spores on the leaf. Percentage of infection was 4.0 to 12.0%, and the periods required for death were 8 to 17 days.

Table 1. Mortality of gypsy moth larvae by *Entomophaga maimaiga* when the larvae were released on a tree trunk to which cadavers were attached

	Released			Non-released
Larval density per cage	25	50	100	25
No. of replicates	4	4	1	1
Percentage of infection ^a	14.0±10.6	7.5±1.5	9.0	0.0
Period required for death (days) ^a	16.1±0.8	15.1±1.6	16.9±1.2	—

^a Mean±SE.

Table 2. Mortality of gypsy moth larvae by *Entomophaga maimaiga* when the larvae were treated with suspensions of materials collected in the field

No. of larvae per plot	Larvae dipped in suspension of			Larvae fed leaves dipped in suspension of			Non-treated
	Cadaver 25	Litter 25	Soil 25	Cadaver 25	Litter 25	Soil 25	
No. of replicates	1	1	1	1	1	1	1
Percentage of infection	12.0	4.0	4.0	8.0	12.0	0.0	0.0
Period required for death (days) ^a	15.3±1.7	17.0	8.0	13.5±6.5	17.3±3.6	—	—

^a Mean±SE.Table 3. Mortality of gypsy moth larvae by *Entomophaga maimaiga* when the larvae were reared with materials collected in the field

	Reared with cadavers			With litter ^b	With soil ^b	Non-treated
	2 hr	24 hr	Entire period			
No. of larvae per plot	5	5	5	5	5	5
No. of replicates	5	5	5	5	5	5
Percentage of infection ^a	8.0±4.9	12.0±12.0	12.0±4.9	44.0±16.0	28.0±10.2	0.0
Period required for death (days) ^a	7.0±0.5	6.3±0.7	12.0±7.0	8.5±0.8	10.1±3.2	—

^a Mean±SE.^b Contact with the materials for entire rearing period.

Results of rearing of the larvae which were kept with the field-collected materials are shown in Table 3. Infection was observed in all treatment plots. Especially, the infection rates were high when the larvae were reared with litter or soil for the entire rearing period; the periods required for death were 6 to 12 days. The variations in mortality and incubation period may be due to the difference in stages of assay animals, rearing temperature, or number of existing spores.

No infection was found in any of the plots of untreated larvae.

In 1986, no infection of this fungus was observed either in release experiments or in treatments with field collected materials.

Resting spores of *E. maimaiga* have been reported to change their internal structure after hibernation and germinate in the spring (AOKI, 1980; KANEDA and AOKI, 1980). The hibernating resting spores were found in egg masses of gypsy moth at the time of outbreak (AOKI et al., 1976). VALOVAGE et al. (1984) successfully infected grasshoppers with germ conidia of *Entomophaga grylli*, a species close to *E. maimaiga* by exposing the insects to its resting spores, although their experiment was performed under laboratory conditions. SPEARE and COLLEY (1912) succeeded in maintaining *E. maimaiga* on gypsy moth larvae for 2 years under outdoor conditions in a box in which the resting spores were lying in the bottom. In their study, the fungus overwintered in the stage of resting spores. They thought, therefore, infection took place during germination of the resting spores formed the previous year. From those findings, it has been spec-

ulated that an epizootic of *E. maimaiga* is initiated by the infection of early instar larvae with hibernated resting spores in the field. In the present study, gypsy moth larvae were infected with the fungus by bringing them into contact with the hibernated resting spores in the field. Therefore, the hibernating resting spores were thought to be a source of the following epizootic. The larvae were infected by bringing them into contact not only with the cadavers of the previous year but also with the litter and soil. This fact suggests that the resting spores which were washed away from the cadavers can also be a source of infection. The results of the present study supported the above hypothesis, and threw more light on the mechanism of an epizootic. There were some differences in larval mortality among the treatments. However, since we could not measure the density of the inoculum, in other words could not know the number of germ conidia attached to each experimental animal, we were able to analyze the results only qualitatively.

Since spores of some fungi such as *Beauveria bassiana* are small and easily blown away by wind, an epizootic in a new host population can be initiated by the migration of spores from other places. On the contrary, germ conidia of *Entomophaga* are so large that it is thought difficult for them to be carried for a long distance by wind. Therefore, if the life of resting spores of *E. maimaiga* is shorter than 2 years, the fungus must alternate its generation every year, and for the prevalence of this disease in a new host population, the migration of infected larvae is prerequisite. In the present experiment the infection with resting spores occurred 1 year after their formation, but not after 2 years. However, since resting spore is a tolerant stage and seems to have a long survival period, there is a possibility that the spores collected after 2 years in the present investigation were alive, although the infection experiments failed. It is, therefore, necessary to clarify conditions for germination of resting spores and their longevity. It is not known in what part of spring the activation of resting spores (germination and consequent formation of germ conidia) starts. In our experiment, activation occurred by April 25 in the southern part of Chiba. The gypsy moth was 1st or 2nd instar larva at that time. Since the early 1st instar larvae of gypsy moth are dispersed by wind, this stage is thought important for the spread of fungus to a new host population. Larvae at this stage are often found on the ground during dispersion, while larvae larger than the 2nd instar are found mostly on trees. This phenomenon may afford more chances for the 1st instar larvae to contact the resting spores than the 2nd or larger instar larvae. In general, epizootics of *E. maimaiga* are conspicuous on the late instar larvae. However, SHIMAZU (unpublished) observed 3rd instar larvae infected with this fungus in the field, and also obtained diseased larvae among these 3rd instar larvae which were collected from the field and reared in the laboratory in May. This confirms that *Entomophaga* epizootics can also occur in the young larval population in the field. We did not use 1st instar larvae in the present experiment, however, as they are susceptible to the conidia of *E. maimaiga* (SHIMAZU and SOPER, 1986); they may also be susceptible to germ conidia. Taking the incubation periods observed in the present experiments into consideration, young instar larvae which were infected with the hibernated resting spores in the field are thought to die and discharge conidia at the same instar or the next. Thus the fungus has two or three opportunities for contagion during the larval period of the gypsy moth. In conclusion, it is most likely that 1st instar larvae are infected with the hibernated resting spores and discharge conidia, which further infect other larvae in the population and make the disease prevalent, causing a heavy epizo-

otic in the old larvae in June and July.

ACKNOWLEDGEMENTS

We wish to thank Professor Keiji KANAMITSU of Nagoya University for his kind permission to use his scientific photograph.

REFERENCES

- AOKI, J. (1980) Dispersion and persistency of entomophthoraceous fungi in an insect population. *Proc. XVI Int. Cong. Entomol., Kyoto, 3-9 Aug. 1980*, p. 208.
- AOKI, J., K. YANASE, T. YANBE and R. KOYAMA (1976) Hibernation of resting spores of *Entomophthora aulicae* in egg masses of the gypsy moth *Porthetria dispar*. *J. Invertebr. Pathol.* **27**: 395-396.
- KANEDA, K. and J. AOKI (1980) Seasonal changes of inner structure of resting spore of *Entomophthora aulicae*. *Proc. Ann. Mtg. Kanto. Div. Jap. Mycol. Soc.*, pp. 8-9 (in Japanese).
- KOYAMA, R. (1954) Two epizootic diseases of gypsy moth. *Shinrin-Boeki* **27**: 296-298 (in Japanese).
- SHIMAZU, M. and R. S. SOPER (1986) Pathogenicity and sporulation of *Entomophaga maimaiga* HUMBER, SHIMAZU, SOPER and HAJEK (Entomophthorales: Entomophthoraceae) on larvae of the gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae). *Appl. Ent. Zool.* **21**: 589-596.
- SPEARE, A. T. and R. H. COLLEY (1912) *The Artificial Use of the Brown-Tail Fungus in Massachusetts with Practical Suggestions for Private Experiment, and a Brief Note on a Fungous Disease of the Gypsy Caterpillar*. Wright & Potter Printing Co., Boston, 31 pp.
- VALOVAGE, W. D., D. R. NELSON and R. D. FRYE (1984) Infection of grasshoppers with *Entomophaga grylli* by exposure to resting spores and germ conidia. *J. Invertebr. Pathol.* **43**: 274-275.