



Storage of Resting Spores of the Gypsy Moth Fungal Pathogen, *Entomophaga maimaiga*

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The fungal pathogen, *Entomophaga maimaiga* causes epizootics in populations of the important North American forest defoliator gypsy moth (*Lymantria dispar*). Increasing use of this fungus for biological control is dependent on our ability to produce and manipulate the long-lived overwintering resting spores (azygospores). *E. maimaiga* resting spores undergo obligate dormancy before germination so we investigated conditions required for survival during dormancy as well as the dynamics of subsequent germination. After formation in the field during summer, resting spores were stored under various moisture levels, temperatures, and with and without soil in the laboratory and field. The following spring, for samples maintained in the field, germination was greatest among resting spores stored in plastic bags containing either moistened paper towels or sterile soil. Resting spores did not require light during storage to subsequently germinate. In the laboratory, only resting spores maintained with either sterile or unsterilized soil at 4°C (but not at 20 or –20°C) germinated the following spring, but at a much lower percentage than most field treatments. To further investigate the effects of relative humidity (RH) during storage, field-collected resting spores were placed at a range of humidities at 4°C. After 9.5 months, resting spore germination was highest at 58% RH and no resting spores stored at 88 or 100% RH germinated. To evaluate the dynamics of infections initiated by resting spores after storage, gypsy moth larvae were exposed to soil containing resting spores that had been collected in the field and stored at 4°C for varying lengths of time. No differences in infection occurred among larvae exposed to fall-collected soil samples stored at 4°C over the winter, versus soil samples collected from the same location the following spring. Spring-collected resting spores stored at 4°C did not go into secondary dormancy. At the time that cold storage of soil containing resting spores began in spring, infection among exposed larvae was initiated within a few days after bringing the soil to 15°C. This same pattern was also found for spring-collected resting spore-bearing soil that was assayed after cold storage for 2–7 months. However, after 31–32 months in cold storage, infections started 14–18 days after soil was brought to 15°C, indicating a delay in resting spore activity after prolonged cold storage.

Keywords: resting spore, *Entomophaga maimaiga*, biological control, cold storage

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INTRODUCTION

Species of Entomophthorales are well known for causing dramatic epizootics in insect populations. These fungi have not been widely developed for pest control because mass production methods are lacking for most species. One technique under investigation is mass production of the environmentally resistant entomophthoralean resting spores (zygospores or azygospores, according to fungal species). Semi-synthetic and completely defined media have been developed for *in vitro* production of resting spores of aphid pathogens in the genus *Conidiobolus* (e.g. Latgé *et al.*, 1978b; Latgé & Sanglier, 1985). More recently, *in vitro* production of *Entomophaga maimaiga* resting spores was demonstrated (Kogan & Hajek, 2000). However, resting spore production is just the first step in the development of these spores for use in biological control since a period of dormancy and possible storage may be required. During these times, resting spores must remain viable and must receive the requirements necessary to satisfy dormancy; only then can germination take place. To investigate whether resting spores are living, vital stains cannot be used because they will not penetrate the thick double wall (T. Finley and A.E.H., unpublished data); therefore, to assess resting spore viability, germination tests or bioassays are necessary.

The Asian fungus *Entomophaga maimaiga* is a virulent pathogen of gypsy moth, *Lymantria dispar*, and has caused dramatic epizootics in North America since 1989 (Hajek, 1999). Due to the effectiveness of this biological control agent there has been interest in its use for biological control. Resting spores (azygospores) appear to be the best stage of *E. maimaiga* for manipulation; resting spores have been successfully used to introduce this fungus to new areas, and use of these spores for inoculative augmentation also appears promising (Hajek, 1999). *E. maimaiga* resting spores undergo obligate dormancy and newly formed spores overwintered in the field in northeastern North America are only able to germinate after mid-March (Weseloh & Andreadis, 1992) or April (Hajek & Humber, 1997). Based on this information and the dormancy requirements for other entomophthoralean resting spores (Hajek, 1997), we hypothesized that *E. maimaiga* resting spores require a cold period prior to germination.

In the present studies, we conducted preliminary investigations to begin to identify optimal conditions for survival of *E. maimaiga* resting spores during dormancy. In initial trials, we evaluated germination of resting spores after overwinter storage under a variety of conditions, including different moisture levels, exposure to sterile and unsterilized soil, light vs. dark, and variable conditions in the field versus at different constant temperatures in the laboratory. Next, we tested resting spore survival after storage across a range of relative humidities. Finally, to understand how to conduct viability assays after storage, alterations in the dynamics of resting spore infectivity after storage at 4°C for varying lengths of time were investigated.

MATERIALS AND METHODS

Experimental Insects

Gypsy moth neonates were obtained from a laboratory colony at the USDA, APHIS, Otis Methods Development Center, MA, USA. Larvae to be used in experiments were reared on a high wheat germ diet (Bell *et al.*, 1981) at 23°C and 14:10 L:D.

General Storage Conditions

In this preliminary experiment, a diversity of storage conditions were evaluated to identify those conditions optimizing *E. maimaiga* resting spore germination. Conditions that were varied included exposure to moisture, light, sterile or unsterilized soil, and ambient temperatures in the field versus several constant temperatures in the laboratory. Late-instar gypsy moth cadavers were collected from the trunks of oak (*Quercus* spp.) trees at Ludlow

Reservoir, Hampden Co., MA, USA during mid-August (1994). Dissections confirmed that cadavers were filled with *E. maimaiga* resting spores. The cadavers were maintained at room temperature (ca. 23°C) in darkness until being allocated to 15 different treatments on 9 September (1994).

For all treatments, 0.7 g of cadavers (ca. 10 cadavers) were placed within each of six 5 × 5 cm envelopes sewn out of landscaping weed control cloth (Rit-A-Weed, AMOCO, Richmond, VA). Envelopes for each treatment were then enclosed together within a larger bag as follows: (1) within a larger landscape cloth bag; (2) within a 3.78 l plastic freezer bag containing a sterile, wet paper towel; (3) plastic bag containing silica gel desiccant (Sigma Chemical); or a plastic bag containing either (4) sterilized or (5) unsterilized soil collected in a mixed species woodlot. An additional treatment (6) consisting of a freezer bag containing unsterilized soil was assembled and the entire freezer bag was covered with aluminum foil to block the light. These bags were placed on the surface of the ground in a mixed species woodlot in Ithaca, NY, USA. The remaining nine treatments were maintained in the laboratory. Six cadaver envelopes containing resting spore-bearing cadavers were either enclosed with a sterile, moistened paper towel, with sterile soil, or with unsterilized soil in freezer bags and one of each treatment was placed at -20°C (7, 8, 9), 4°C (10, 11, 12) and 20°C (13, 14, 15). All soil included in the treatments was at ca. 30% water content. Soil moisture was quantified as % soil moisture = (wet weight of soil - dry weight of soil)/dry weight of soil. For all treatments maintained at fixed temperatures in the laboratory, plastic bags were covered with foil to block light. At monthly intervals during the study, silica gel was replaced if wet and bags were checked to assure they were closed; the moistened paper towel remained moist throughout the storage period.

To determine effectiveness of treatments, the viability of the resting spores was determined at 7, 7.5 and 8 months after the start date. For each assay, one envelope of cadavers was removed from each treatment. Cadavers within each envelope were placed into a 15 ml centrifuge tube and allowed to soak for 1 h in deionized (DI) water. Cadavers were then broken up by vortexing and macerating with a glass rod, if needed. Using DI water for rinsing, solutions were sieved through a 63 µm sieve to remove debris and resting spores were then collected on a 20 µm sieve. Resulting collections of resting spores were then pelleted by centrifugation. Spores were soaked in 5 ml of a 30 ppm mercuric chloride solution for 5 min prior to five DI water washes. Mercuric chloride is a biocide but in low concentrations was thought to have little effect on entomophthoralean resting spores due to their thick outer walls (e.g. Perry *et al.*, 1982b); because resting spores germinate slowly over many days, preventing contamination of Petri dishes is critical to evaluating germination.

Resting spore germination was tested by plating 0.5 ml of a suspension of resting spores in sterile DI water with 5 µl Gentocin (Schering-Plough, Kenilworth, NJ, USA) onto 1.5% water agar in a 100 mm diameter Petri dish, with resulting densities of ca. 100 resting spores mm⁻². Four replicate Petri dishes per sample were plated and then maintained at 15°C, 14:10 light:dark (L:D). Percent germination was determined by noting the activity of ten resting spores in each of 10 fields per plate at ×50 magnification on a dissecting microscope. At each of the three evaluation dates, counts were made every second day for a total of 12–14 days. This experiment was also repeated between 8.5 and 9 months after the start date. Data from this last assay date were not included in overall analyses because for many treatments, remains of empty resting spores were abundant and percent germination had drastically declined. For this last repetition, all germination had occurred by 6 days, while for earlier repetitions, germination generally occurred over 12 days.

Relative Humidity

Based on previous results, an experiment was conducted to determine which relative humidities (RH) optimize resting spore storage. Specific RHs tested were 0, 14, 35, 58, 76, 88 and 100%. Chemicals used to generate atmospheres of each RH were a silica gel desiccant, or saturated solutions of lithium chloride, magnesium chloride, magnesium nitrate,

sodium chloride, potassium chloride and water, respectively (Budavari, 1989). One micro-environment per RH was constructed using a 0.9 l glass jar with 100 g (dry weight) of the respective chemical in the base; the RH in each jar was initially monitored and jars were allowed to equilibrate for two days before placing samples inside. Seven Tyvek envelopes (ca. 8 × 10 cm) were constructed and 0.7 g of late instar gypsy moth cadavers known to contain *E. maimaiga* resting spores were placed into each envelope. Cadavers used in this experiment were collected in June (1997) from an epizootic in Oakland Co., MI, USA just prior to the experimental set up. Envelopes were suspended above the respective chemicals in each of the seven glass jars. All jars were then placed at 5°C in the dark. Resting spore germinability was tested 9.5 months later by counting germinating resting spores over six weeks. Resting spore germination was determined using the same protocol as above, except that 0.1 g of cadavers were removed from the envelopes for each germination test and allowed to soak in DI water for 10 min, with three replicate Petri dishes containing resting spores monitored per treatment. Tests were repeated with and without mercuric chloride washes. Counts were made at 14 time points over the ensuing six-week period.

Infection Dynamics After Storage

Resting spore-bearing soil was collected along Elliot Springs Run and Archer Run, eastern slope of Great North Mountain, Deerfield Ranger District, George Washington National Forest, VA, USA on 16 December (1996) and 12 April (1997). The organic layer of the soil was collected from three locations around the bases of each of five trees per date, within 10 cm of trunks and at a depth of ≤ 3 cm. Soil samples collected at these locations on 12 April (1997) contained 7625 ± 1171 resting spores g^{-1} of dry soil (counts made using the method of Hajek and Wheeler, 1994). We hypothesized that the resting spores were between 0.5–1.5 years old in the December collection, being deposited in the soil from cadavers of gypsy moth larvae killed by *E. maimaiga* when this plot experienced dramatic decreases in high density gypsy moth populations during the two previous field seasons. Soil was immediately shipped to Ithaca, NY, USA and stored at 4°C until use.

Bioassays were initiated on 20 May (1997) when some of the soil from each site, collected in December and April, was brought out of the cold. For these initial assays, we used moisture levels of the soil as collected from the field and determined the soil moisture to be $74 \pm 1.6\%$ (mean \pm SE) with a pH of 6.4–6.6. Soil was mixed well and 30 g (wet weight) of each soil sample were placed into each of three 4.5 × 10.5 cm diameter polypropylene deli containers. The containers were placed into a 15°C, 14:10 L:D incubator. Ten early fourth instar gypsy moth larvae were placed onto the soil in each cup. After four days, larvae were removed from cups, placed individually into 29 ml plastic cups containing 10 ml high wheat germ artificial diet (Bell *et al.*, 1981) and monitored daily for 10 days, checking for mortality and *E. maimaiga* conidial production. Mortality initiated by *E. maimaiga* resting spores results in conidial production (Hajek, 1997), therefore larvae that perished but did not exhibit *E. maimaiga* conidiophores were not considered to have been infected. This comparison of soil collected in December vs. April was repeated beginning 26 May and 7 June (1997), using the same soil containers.

To evaluate changes in resting spore activity after prolonged cold storage, only resting spore-bearing soil samples collected in April (1997) along Elliot Springs Run were used. Our principal objective was to determine how long resting spores would initiate infections after being stored. Similar procedures were used to test samples although, based on results from other studies, moisture of this highly organic soil was brought to near field capacity (200% wet weight) and 14 g of soil (wet weight) were used per containers. For the first exposure period, soil was transferred to 15°C on 2 June (1997) and larvae were placed on the soil on 6 June. Using the same soil containers, larvae were then exposed for eight additional intervals, with exposures ending 28 July. This experiment was repeated monthly, bringing fresh soil out of the cold each month, until February (1998; 10 months after soil collection). Each month from July–February, bioassays were initiated 0–2 days after soil

was brought to 15°C. The soil samples warmed each month were used for bioassays until no further infections occurred, resulting in 5–8 bioassays for each soil sample.

Laboratory observations suggested that with increasing time in cold storage, once brought to 15°C, resting spores required longer to begin germinating. To document this, beginning 31 months after the April (1997) soil collection, bioassays were again conducted using the procedure above. Larvae were exposed during nine four-day intervals. This was repeated the next month for eight four-day exposure intervals.

Infection from resting spore-bearing soil was compared after 10 months of storage, using the same procedures but at 10, 15, 20 and 25°C and 14:10 L:D. Larvae were exposed to soil for four day intervals beginning 0, 8, 14 and 28 days after soil was moved to the temperatures being evaluated.

Data Analysis

To evaluate differences among general storage treatments and storage at different relative humidities, arcsine-transformed percentage germination was analyzed using two-way analysis of variance. For the analysis of general storage treatments, the interaction between experimental replicate and treatment was significant, so data were analyzed separately by date. Posthoc comparisons with the Sidak inequality were used to partition the overall α of 0.05 (Jones, 1984).

RESULTS

General Storage Conditions

For resting spores maintained in the laboratory, germination was only detected among samples stored at 4°C and not 20 or –20°C (Table 1). Only those resting spores stored at 4°C with either sterile (11) or unsterilized (12) soil germinated while those resting spores stored at 4°C with a moistened paper towel (10) did not germinate. Resting spores bagged with sterile or unsterilized soil germinated at equivalent levels, which were mostly lower than the other field-maintained treatments that germinated (Table 1). Temperatures experienced in the field were more variable than the constant temperatures in the laboratory but on average

TABLE 1. Percent germination of *Entomophaga maimaiga* resting spores stored in different ways in the field and laboratory for 7–8 months

Treatment (number)	% Germination (mean \pm SE) ^a		
	Assays begun 3 April	19 April	4 May
<i>Field</i> ^{b,c}			
On the soil (1)	11.5 \pm 1.6 b	15.0 \pm 1.2 b	24.3 \pm 3.4 ab
In plastic bag with a sterile, moistened paper towel (2)	17.5 \pm 0.3 ab	15.8 \pm 3.9 ab	34.8 \pm 2.7 a
In plastic bag with sterile soil (4)	24.0 \pm 3.0 a	25.3 \pm 3.1 a	20.3 \pm 3.6 b
In plastic bag with unsterilized soil (5)	0.5 \pm 0.3 c	18.5 \pm 1.9 ab	8.8 \pm 0.3 c
In plastic bag with unsterilized soil, dark (6)	13.3 \pm 1.7 b	14.3 \pm 1.1 b	16.5 \pm 1.3 bc
<i>Laboratory</i> ^{b,d}			
4°C, in plastic bag with sterile soil, dark (11)	0 \pm 0 c	0.3 \pm 0.3 c	18.3 \pm 1.6 bc
4°C, in plastic bag with unsterilized soil, dark (12)	0 \pm 0 c	0 \pm 0 c	14.8 \pm 1.0 bc

^aMeans followed by different letters are significantly different ($P < 0.05$). 3 April assay: $F = 43.37$, 19 April assay: $F = 19.25$, 4 May assay: $F = 12.62$.

^bSoil added to bags was at ca. 30% moisture content.

^cResting spores stored on the forest floor in a plastic bag with desiccant did not germinate (3).

^dAll resting spores held at –20°C (7, 8, 9) and 20°C (13, 14, 15) and those held at 4°C in bags with a sterile, moistened paper towel (10) did not germinate.

were similar to the 4°C treatment, with an average ambient temperature from September through March of $3.5 \pm 2.8^\circ\text{C}$ (monthly averages ranging from -6.7 to 15.2°C (NOAA, 1994, 1995)).

The highest germination occurred for those resting spores maintained in the field in plastic bags containing either sterile soil (4) or a sterile moistened paper towel (2) (Table 1). Resting spores on top of the ground in a porous landscape cloth bag (1) germinated at an intermediate level compared with other treatments. Resting spores in bags containing unsterilized soil (5) germinated less than resting spores in bags with sterile soil (4) for two of the three assays. Comparing germination of resting spores stored with unsterilized soil, either with or without light, demonstrated that lack of light during the overwintering period did not consistently stimulate or inhibit resting spore germination (Table 1); clearly, light is not needed during storage for subsequent germination. For resting spores maintained in the field over the winter, the only samples not germinating were those bagged with desiccant. Interestingly, overall per cent germination increased with time for repetitions initiated seven through eight months after the start date (7 months = $9.5\% \pm 3.6$, 8 months = $19.6\% \pm 3.1$) ($t = 2.60$; $P < 0.05$).

For the repetition of this experiment conducted 8.5–9 months after initial storage, resting spores in the landscape cloth bag directly on the soil in the field were still germinating well ($19.3 \pm 3.0\%$) although all germination was seen within four days of setting up the germination assay. Germination of resting spores stored in the field in plastic bags containing unsterilized soil in light ($6.8 \pm 2.0\%$) and dark ($3.8 \pm 1.6\%$) or a sterile, moistened paper towel ($2.8 \pm 0.9\%$) were the only other treatments in which any germination was observed at the 8.5–9 month assay.

Relative Humidity

Resting spores stored at 88 and 100% RH for 9.5 months did not germinate. For all resting spores, maximal levels of germination were found for resting spores stored at 58% RH (Table 2). Intermediate germination occurred for resting spores stored at 76, 14 and 0% RH and few resting spores stored at 35% RH germinated. While pretreatment with mercuric chloride was useful for killing contaminants during germination tests, resting spore germination was higher when it was not used ($23.5 \pm 5.8\%$) compared with when it was used ($15.5 \pm 3.2\%$). Total germination was close to significantly different between treatments with or without mercuric chloride ($P = 0.0556$).

TABLE 2. Total percent germination (\pm SE) of *Entomophaga maimaiga* resting spores from gypsy moth cadavers collected in June and stored at different relative humidities at 4°C for 9.5 months

Relative humidity ^a	% Germination Mean \pm SE ^b
0%	17.7 ± 2.7 bc
14%	8.3 ± 1.8 bc
35%	2.7 ± 1.2 c
58%	44.0 ± 9.6 a
76%	25.0 ± 3.2 ab

^aResting spores stored at 88 and 100% RH did not germinate.

^bMeans followed by different letters are significantly different ($t = 3.08$; $P < 0.05$). Data from resting spores with and without mercuric chloride washes before germination tests were merged for analysis.

Infection Dynamics After Storage

Percent infection of gypsy moth larvae did not differ between resting spore-bearing soil collected in December (1996) and stored at 4°C and resting spore-bearing soil collected at the same sites in April (1997) and stored at 4°C ca. 5 weeks ($F = 0.86$; $P > 0.05$).

Resting spore-bearing soil collected in April (1997) stored at 4°C and used for bioassays beginning in June caused infection over 3–5 weeks after being warmed to 15°C. Monthly bioassays performed from June (1997) through February (1998) demonstrated similar patterns, with infection levels usually > 20% for the first 3–4 day larval exposure period, beginning 0–4 days after soil was warmed followed by an increase in infection after the first exposure period (Figure 1; data from 5 months shows typical pattern). Spring-collected resting spores therefore remained at the same readiness to germinate over 10 months of cold storage, while in the field, resting spores would have ceased germinating in early summer (Hajek & Humber, 1997). The pattern of infection dynamics changed after soil had been stored at 4°C for > 31 months. Instead of immediate infection when larvae were exposed to soil, only those larvae exposed ≥ 14 days after the soil was brought to 15°C became infected. The duration of infection was similar, lasting between four and five weeks (Figure 1).

Resting spores stored in the cold for ca. 10 months did not infect larvae at 25°C (Figure 2). For resting spores stored at 10, 15 and 20°C, infections were close to being significantly different by temperature ($F = 3.12$; $P = 0.0627$) and the interaction between temperature and length of exposure was significant ($F = 10.53$; $P < 0.05$). The most striking difference was that at 20°C, infections occurred only during the first two exposure periods and at relatively lower levels (Figure 2). Resting spores maintained at 10°C did not infect immediately, although high levels of infection occurred during the 28–32 day exposure period.

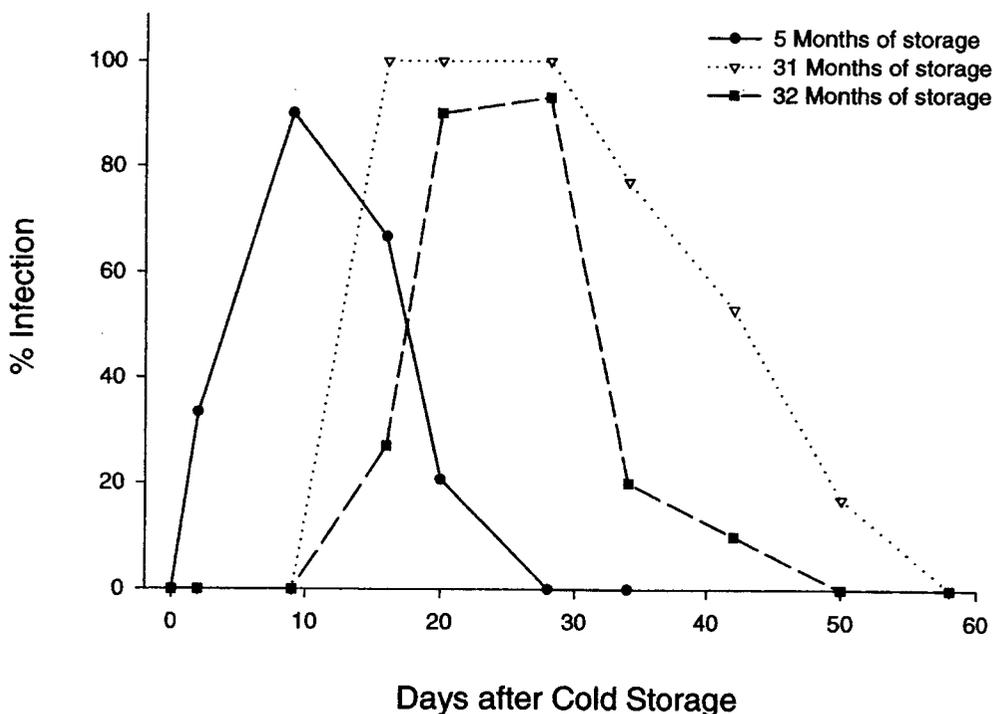


FIGURE 1. Percent infection of gypsy moth larvae exposed to soil containing *Entomophaga maimaiga* resting spores at 15°C, after varying lengths of cold storage. Mid-points of four-day exposure periods are plotted on the x-axis.

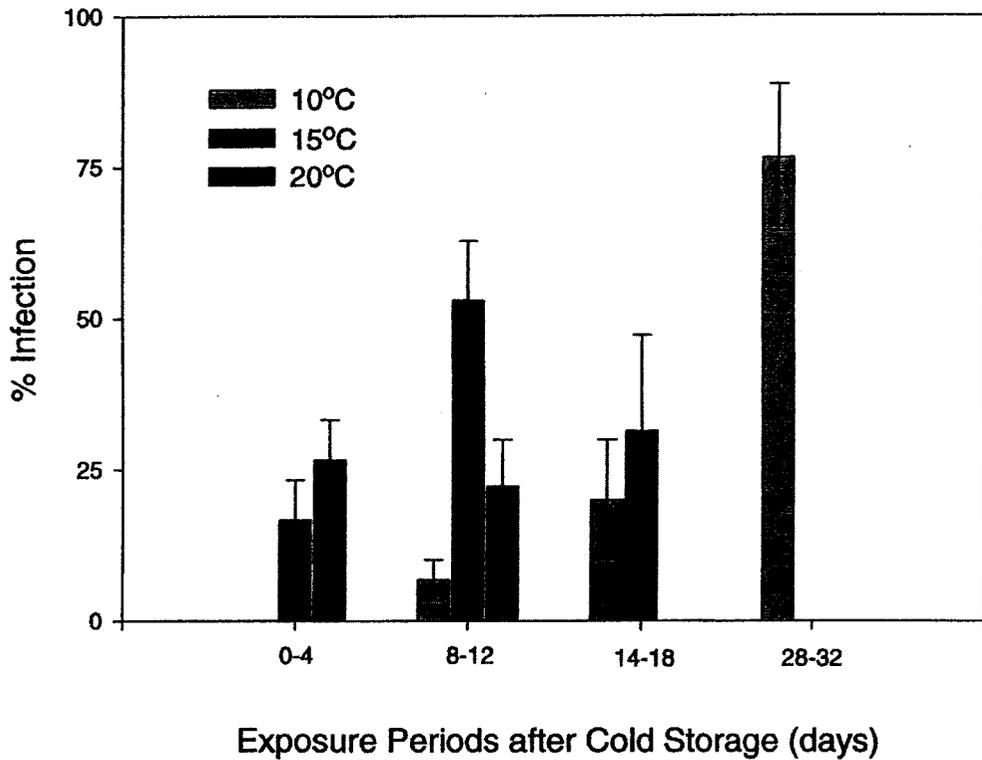


FIGURE 2. Percent infection of gypsy moth larvae exposed to soil containing *Entomophaga maimaiga* resting spores at different temperatures, with successive four-day exposure periods after cold storage of resting spores for 10 months.

DISCUSSION

We determined that both temperature and moisture during storage influenced survival and subsequent germination of *E. maimaiga* resting spores. Resting spores did not germinate after exposure to freezing temperatures for seven months. This was not unexpected as resting spores of *Zoophthora radicans* died after exposure to -20°C for prolonged periods (Perry & Fleming, 1989). Resting spores of the aphid pathogen *Zoophthora canadensis* in cadavers above the snowline in Ontario, Canada did not survive the winter although those below snowline were able to germinate after winter (Perry *et al.*, 1982a); because resting spores of *Z. canadensis* can survive dry conditions, the authors suggested that the increased cold above the snowline was responsible for resting spore mortality. In contrast, resting spores of three species of *Conidiobolus* exposed to -30°C for 18 months were unaffected by such treatments (Krejzova, 1971).

As hypothesized, *E. maimaiga* resting spores germinated after a cold period. The dormancy requirements for resting spores of several species of entomophthoralean fungi have been identified as several months of temperatures near 0°C . However, for *Z. canadensis* in Canadian forests, long photoperiods are needed to satisfy dormancy (Hajek, 1997). To be competent to germinate, *E. maimaiga* resting spores did not need long photoperiods. During this study, we also discovered that resting spores in cadavers collected from the field in June and immediately placed at 4°C germinated successfully the following spring. Therefore, although newly produced resting spores in the field would receive an aestival period of

warmth plus long photoperiods before colder temperatures and shorter photoperiods, aestivation was not required for resting spore germination the following spring.

Overwintering at the soil surface in the field was superior to exposure to constant temperatures in the laboratory. We included exposure to soil as a treatment in this study because the presence of soil was associated with increased germination of resting spores of the entomophthoralean aphid pathogen *Conidiobolus obscurus* (= *Entomophthora obscura*) (Latgé *et al.*, 1978a). Exposure to soil was not necessary during storage and, in fact, unsterilized soil was associated with lower germination than sterile soil. This result could be due in part to potential fungal hyperparasitism or attack of germinating resting spores by bacteria (Perry *et al.*, 1982a).

High moisture was detrimental during the overwintering period as *E. maimaiga* resting spores did not germinate when stored at 88 or 100% RH. Moisture relations associated with resting spore longevity for other entomophthoralean species are variable; for many species, resting spores that are air-dried for more than several weeks do not survive (Perry *et al.*, 1982a), although *Conidiobolus thromboides* (= *Entomophthora virulenta*), *C. obscurus*, (= *Entomophthora thaxteriana*), *Conidiobolus destruens* (= *Entomophthora destruens*), and *Z. canadensis* can be stored dry for several years (Krejzova, 1973). *C. obscurus* resting spores also can be stored at 100% RH and 4°C for one year, preferably in a mixture with clay and water (Perry & Latgé, 1982).

In the field, resting spores in soil infect gypsy moth larvae from late April or early May through mid-June, but not all resting spores germinate each year (Hajek *et al.*, 1995; Hajek & Humber, 1997). After this 'germination window', resting spores that did not germinate are not able to germinate until the following spring because they undergo a secondary dormancy. However, when resting spores that were ready to germinate were collected in soil during early spring and stored at 4°C, they did not go into a secondary dormancy. We cannot say what factors are responsible for cessation of germination in the field in June but the spring-collected resting spores placed in the cold did not receive this stimulus causing a cessation of germination. In fact, while at 4°C, the resting spores remained ready to germinate for more than 31 months. However, after cold storage for more than 31 months, resting spores did not germinate immediately upon warming to 15°C, instead requiring ≥ 14 days at 15°C before causing infections. This delay in infection could result from the increasing age of the spores or, conversely, the prolonged exposure to 4°C.

After dormancy, we know that soil moisture is important for resting spore germination (Hajek, 1999). Temperature also influences germination because we found numerically greater activity of *E. maimaiga* resting spores at 15°C during the first 18 days of assays although by the 28–32 day exposure, infection at 10°C was high and resting spores at other temperatures had completed germination. Germination of *C. obscurus* resting spores was optimal at 15°C, lasting for about three weeks (Latteur *et al.*, 1982), with no pronounced delay before germination increased at 10°C. In contrast, for *E. maimaiga* a delay occurred before resting spores at 10°C became very active. The temperature range allowing germination of *Z. radicans* resting spores changed from 8–20°C at three months to 4–32°C after eight months of storage at 4°C (Perry & Fleming, 1989). Mean germination time and maximum germination for *Z. radicans* resting spores decreased as poststorage temperature increased. In contrast to results from the present study, Perry and Fleming (1989) also found that as *Z. radicans* resting spores aged from 5 to 10 months, the variance in spore germination time increased significantly.

To use *E. maimaiga* resting spores for biological control, storage conditions must be identified for satisfying resting spore dormancy as well as maintaining resting spore viability and subsequent ability to germinate. Based on this study, optimal conditions during storage would include an intermediate moisture level and a cold period during storage, e.g. 4°C but not –20°C. The present studies were conducted without knowledge of the minimum length of time at cold temperatures necessary to satisfy dormancy of *E. maimaiga* resting spores and such studies are presently being conducted. We hope that this information can then be

applied to developing techniques for optimal handling of *E. maimaiga* resting spores for use in biological control. The longevity of entomophthorean resting spores would certainly be an asset for a biological control agent. *C. obscurus* resting spores stored dry at 7–15°C survived at least 6.5 years (Krejzova, 1973). Field-collected soil bearing *E. maimaiga* resting spores show a similar trend, causing infection after five years storage at 4°C (A.E.H., unpublished data).

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