

Nondormancy in *Entomophaga maimaiga* azygospores: effects of isolate and cold exposure

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Abstract: Azygospores (resting spores) of the fungal pathogen *Entomophaga maimaiga* are produced in later larval instars of the gypsy moth *Lymantria dispar* and normally enter constitutive dormancy. In the laboratory cadavers of recently dead larvae containing immature azygospores were placed on 1.0% water agar at 20 C for 2 wk after host death, allowing time for azygospore maturation. We found that some azygospores produced in this way did not enter dormancy. To investigate nondormancy, suspensions of azygospores from individual cadavers were transferred to moist, sterile soil at 15 C. Groups of gypsy moth larvae were exposed sequentially to azygospores for consecutive 4 d periods for 196 d. Infections first were seen among larvae exposed 24–28 d after bioassays began, and infection potential continued 196 d. Germination tests confirmed azygospore germination. Additional cadavers containing azygospores produced under the same conditions were maintained at 4 C for 1–8 mo, and each month new sequential bioassays were initiated. There was a general trend of earlier initiation of infection with longer durations of 4 C exposure; after 6–8 mo at 4 C first infections occurred 6–10 d after bioassays began. With 5–8 mo at 4 C infection levels declined after 96 d of sequential bioassays. Activity of azygospores differed by the individual larval cadaver in which they were produced; azygospores from 29.2% of cadavers yielded only 0–0.3% infection. Infection from nondormant azygospores did not differ among three

Japanese isolates and two North American isolates, although azygospores from a third North American isolate caused no infections in 84 d, suggesting that dormancy had not been prevented.

Key words: biological control, Entomophthorales, gypsy moth, *Lymantria dispar*, spore dormancy, Zygomycota

INTRODUCTION

Fungal species in order Entomophthorales can cause extensive epizootics that control outbreaks of specific insect and mite pests (Pell et al 2001). Researchers and land managers are interested in the potential for mass production of Entomophthorales for dissemination of these fungi as biological control agents. However entomophthoralean species are generally fastidious obligate pathogens and methods for mass production have not been straightforward. Entomophthoralean species generally produce two types of spores: short-lived, relatively fragile conidia and long-lived, thick-walled resting spores (zygospores or azygospores). Some research has focused on mass production of resting spores, which can be produced in vitro for some species (Pell et al 2001). However resting spores typically enter constitutive dormancy after being produced and occurrence of this dormancy is a major obstacle to large scale application of these spores for biological control (i.e. after azygospore production there is a long period before these spores begin to germinate).

While zygospores of one species of Entomophthorales, the aphid pathogen *Conidiobolus thromboides* (= *Entomophthora* sp. nr. *thaxteriana*), does not enter dormancy (Soper et al 1975), for the remainder of entomophthoralean species that have been studied azygospores and zygospores enter constitutive dormancy after they mature within host cadavers (Hajek 1997a). Termination of dormancy has been the focus of numerous studies (Hajek 1997a), and factors associated with the termination of dormancy for several entomophthoralean species include prolonged cold or long photoperiods. When resting spores no longer are dormant they germinate asynchronously to eject one to several infective germ conidia.

The entomophthoralean species *Entomophaga maimaiga* infects larvae of the gypsy moth, *Lymantria dispar*, a major defoliator of northeastern U.S. forests.

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TABLE I. Isolates of *Entomophaga maimaiga* used to evaluate azygospore germination

ARSEF No. ¹	CU No. ¹	Collection location	Collection year
5384	96MD1-2F	Sudlersville, Queen Anne's Co., Maryland	1996
6051	98MA2-4A	The Notch, Hampshire Co., Massachusetts	1998
6162	98JP2-9A	Chonan, Chiba Pref., Japan	1998
6253	98JP2-4A	Chonan, Chiba Pref., Japan	1998
6265	98JP3-8B	Tsukuba, Ibaraki Pref., Japan	1998
6625	00NYI-1-2	Yellow Barn St. For., Tompkins Co., New York	2000
7105	01JP4-12-2	Morioka, Iwate Pref., Japan	2001

¹ ARSEF = USDA, Agricultural Research Service Collection of Entomopathogenic Fungal Cultures; CU = Cornell University, Hajek lab.

Gypsy moth is native to Europe, northern Africa and temperate Asia, whereas this fungal pathogen is native to Japan, northeastern China and the Russian Far East. *E. maimaiga* that originated from Japan is now established in northeastern North America throughout most of the range of gypsy moth (Nielsen et al 2005). *E. maimaiga* can provide effective control of gypsy moth (Hajek 1999), and inoculum is in demand by land managers trying to prevent the defoliation and nuisance caused by outbreak populations of gypsy moth.

In vitro production of *E. maimaiga* azygospores is possible (Kogan and Hajek 2000) but understanding how and when azygospores might germinate has been more difficult. In the field later instar gypsy moth larvae infected by *E. maimaiga* frequently die hanging onto tree trunks in late Jun or early Jul, and azygospores develop within the cadavers. Cadavers fall from trees (Hajek et al 1998b), decompose and azygospores persist in the upper layers of soil, especially around bases of trees (Hajek et al 1998a). In the field *E. maimaiga* azygospores begin to germinate after being in the soil under ambient conditions for ca. 9–10 mo (Hajek and Humber 1997). Germination lasts only about 1.5–2 mo in the field. When an *E. maimaiga* azygospore germinates a germ tube grows upward and one germ conidium, produced at the end, is ejected into the air. If the germ conidium does not contact a host it can produce and discharge a secondary germ conidium to allow another chance to reach a host. Each year many *E. maimaiga* azygospores do not germinate but contribute to a reservoir of azygospores in the soil (Weseloh and Andreadis 1992, Hajek and Humber 1997). If *E. maimaiga* azygospores intended as a microbial control product require 9–10 mo before only some will germinate, the use of these spores as a biological control product would require major changes in the typical procedures for biopesticide use.

Our laboratory has identified a method to prevent azygospore dormancy in species *E. maimaiga*, for

which it is assumed that constitutive dormancy always occurs in the field (Hajek and Humber 1997). In the present paper we describe conditions under which nondormant azygospores were produced in the laboratory. Bioassays were used to evaluate azygospore activity because this type of assay is more successful and reproducible than germination tests, although limited germination tests were used to confirm azygospore activity. Consecutive bioassays at 15 C were conducted for at least 196 d to evaluate the duration of nondormant azygospore activity. To understand whether at least some azygospores remain nondormant and active after cold exposure, nondormant azygospores were placed at 4 C for 1–8 mo. Each month bioassays were initiated with aliquots of azygospores transferred from 4 C to 15 C. The ability of azygospores of *E. maimaiga* isolates from Japan and North America to germinate without undergoing dormancy was compared.

MATERIALS AND METHODS

Production of nondormant azygospores.—Gypsy moth larvae were obtained as neonates from the USDA, APHIS, Otis Methods Development Center, Otis Air National Guard Base, Massachusetts, and reared on high wheat germ artificial diet at 23 C, 60% RH and 14:10 (L:D) (Bernon 1995).

To produce *E. maimaiga* azygospores isolates from both North America and Japan were used (TABLE I). *E. maimaiga* protoplasts were grown in 9.5 mL Grace's insect cell culture medium plus 0.5 mL fetal bovine serum (GIBCO/BRL, Gaithersburg, Maryland). Late fourth- and early fifth-instar gypsy moth larvae were injected at the base of a proleg with 10 μ L of a 1×10^5 per mL suspension of *E. maimaiga* protoplasts (=injection larvae) (Soper et al 1988). After injection larvae were reared individually on artificial diet in 29.6 mL clear plastic cups at 20 C and 14:10 (L:D) and checked daily for mortality. Azygospore production was quantified per cadaver and averaged $2.79 \times 10^6 \pm 2.71 \times 10^5$ (n = 47) for isolate ARSEF 6162.

Types of assays.—*Azygospore germination.* Studies were conducted to confirm that azygospores were germinating

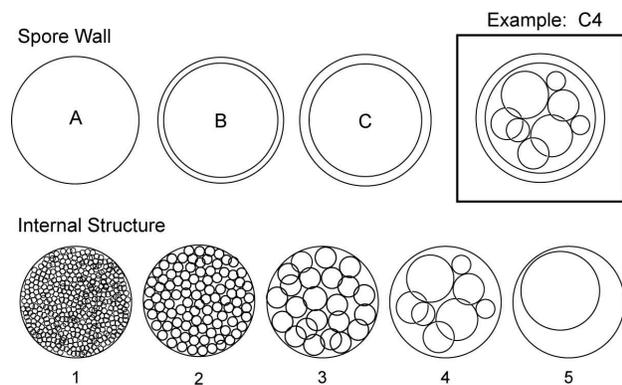


FIG. 1. Stages in maturation of *Entomophaga maimaiga* azygospores. Numbers denote internal structure and letters denote wall structure. Normal resting spores are thin-walled with granular interiors when initially formed (=A1) and after complete maturation have a thickened (double) wall with several to only one internal lipid droplet (=C4 and C5).

without a period of constitutive dormancy. After injection larvae were maintained at 15 C and 14:10 (L:D) for 6 d, after which cadavers were moved individually to 1.5% water agar in 29.6 mL clear plastic cups. Two weeks were considered necessary for azygospore maturation, and cadavers were maintained in cups at 15 C for seven additional weeks. Cadavers from injected larvae were dissected and their contents viewed at 200 \times to confirm the presence of azygospores. Eleven azygospore-containing cadavers were soaked individually in distilled water for 18 h at 15 C. Azygospores then were washed through a 63 μ m sieve and collected on a 20 μ m sieve. Following procedures reported in Perry et al (1982) azygospores were soaked in 30 μ g/mL mercuric chloride 5 min and rinsed five times with sterile distilled water before 0.6 mL azygospore suspension was spread on 1.5% water agar in 100 mm Petri dishes, with 12.5 μ L 100 mg/mL gentamycin sulfate added to each Petri dish. This resulted in a thin film of a azygospore suspension containing water on the water agar surface. Azygospore densities averaged 50.3 ± 11.6 per mm^2 for germination plates from nine larval cadavers. Plates were incubated at 15 C, 14:10 (L:D), and the development of azygospores was observed every other day for 12–14 d. Following scoring from Hajek and Humber (1997) azygospores were recorded as fully germinated if they produced a germ tube from which a germ conidium was ejected.

Quantifying developmental stages of azygospores. After hosts die from *E. maimaiga* infections hyphal bodies within cadavers round up and develop a double wall while the contents change from granular to one to several lipid droplets (Hajek and Humber 1997). Expanding on the system of Latgé (1980) to document morphological variability, we quantified resting spore maturation based on wall development and internal characteristics (FIG. 1); for full maturation, cells progress from A1 to C4–C5. These changes begin soon after host death and for some cells are complete 2 d later. However, in one study Hajek and Humber (1997) reported that >35% of cells within cadavers had not reached C4–C5 by 5 d after host death. In addition cells do not always

progress to the C4–C5 stage (AEH unpubl). In the present study we assumed all azygospores had completed development by 13–14 d after host death. To quantify developmental status the morphology of 10 azygospores from 10 fields of view at 200 \times was rated using FIG. 1.

General bioassay protocol. To evaluate nondormancy azygospores were produced in gypsy moth larvae in the laboratory. Bioassays consisted of exposing naïve gypsy moth larvae (=exposure larvae) to azygospores added to autoclaved soil. Negative controls (larvae exposed to autoclaved soil) conducted in previous studies have never become infected (AEH unpubl). Exposure larvae were maintained in containers with azygospores for 4 d intervals and were then reared to detect infection. If exposure larvae become infected (via germ conidia) this indicated that azygospores had not entered dormancy.

Nondormancy assays with ARSEF 6162. To produce nondormant azygospores for studies of activity with and without 4 C exposure, larvae were injected with ARSEF 6162 and maintained at 15 C in the dark. Six days after injection (ca. the date of host death) 60 cadavers from injected larvae were placed individually in 29.6 mL plastic cups containing 10 mL 1.0% water agar at 20 C in the dark to allow 14 d for resting spore maturation. Three cadavers (=three samples) were used as the sources for azygospores for basic studies and the rest remained on water agar and were placed at 4 C.

Basic activity of nondormant azygospores. Following the procedure below groups of exposure larvae were exposed sequentially to azygospores, beginning 14 d after host death. To conduct bioassays surface soil was collected from the bases of red oaks (*Quercus rubra*), pooled and autoclaved. Approximately 20 gm soil were placed in each of three clear plastic containers with lids (11 cm diam \times 4.5 cm tall). The three cadavers not placed at 4 C were removed from water agar and smears of their body contents were checked microscopically at 200 \times to confirm that they contained C4 + C5 azygospores. Cadavers were soaked individually in 5 mL tap water and gently macerated with a glass rod. The suspension from each cadaver was poured into one soil cup, adding water if necessary so that the soil was moist but not saturated. Ten early fourth-instar exposure larvae were added to each soil dish, which was tightly capped and placed at 15 C, 14:10 (L:D). After 4 d the exposure larvae were removed from each soil cup. By 4 d any exposure larva that had become infected would not yet have died and sporulated so any infections that subsequently resulted were initiated by germ conidia produced from azygospores on the soil and were not caused by cross infection. Exposure larvae were placed in individual 29.6 mL plastic cups containing artificial diet at 20 C, 14:10 (L:D) and monitored daily for 10 d to record mortality and conidiation. Production of conidia from later instar exposure larvae is an additional indication that azygospores initiated infection (Hajek 1997b). Dead exposure larvae were maintained seven additional days to allow azygospore development and were stored at 4 C, followed by dissection and microscopic examination at 200 \times to detect azygospore production.

For each individual sample, bioassays were repeated 2 d after exposure larvae were removed from the soil, using the same cups of soil; this procedure continued for a total of 33

bioassays (i.e. bioassays ended 196 d after initiation). Throughout this period we removed frass from the soil surface between bioassays to retard growth of saprophytes and added water when necessary to keep the soil moist.

Activity of nondormant azygospores after cold exposure. To investigate the effect of cold exposure on nondormancy, every month for 8 mo after placement of cadavers at 4 C azygospores from three cadavers (from injected larvae) were used for bioassays. Cadavers were brought to room temperature and treated as described above to add the azygospores from within the cadavers to soil cups and sequentially expose naïve larvae to them. With subsequent batches of exposure larvae maintained in the same soil cups some mortality due to septicemia was noted, so further bioassays were conducted with the following method. Beginning with the azygospores that had been stored at 4 C for 3 mo, azygospores from three cadavers were treated with mercuric chloride (see method above) before being placed on soil. Azygospores from an additional three cadavers were not treated with mercuric chloride washes, as for the assays before 3 mo of 4 C exposure. For each sample of azygospores that had been exposed to 4 C, a total of 35 sequential bioassays were conducted (i.e. bioassays ended after 208 d).

Effect of origin of fungal isolates on nondormant azygospore activity. Similar bioassays were conducted to test whether nondormant azygospores could be produced with different fungal isolates. Azygospores were produced with three isolates from North America (ARSEF 5384, 6051, 6625) and three from Japan (ARSEF 6253, 6265, 7105) (TABLE I). ARSEF 6162, as used previously, grew poorly and was not suitable for continued study. Therefore ARSEF 6253, isolated on the same date from the same location, was substituted. To produce azygospores, injected larvae were maintained at 23 C with a photoperiod of 14:10 (L:D) for 9 d. The cadavers resulting from injections then were placed in 29.6 mL plastic cups containing 1.0% water agar at 20 C in the dark. After 13 d cadavers of insects injected with each isolate were chosen for bioassays. When choosing which cadavers to add to soil cups, fungal cells in cadavers were checked microscopically. We specifically chose cadavers with most azygospores in the C4 or C5 stages. Differing resting spore development status by isolate was noted as the study progressed. Therefore, when bioassays were begun with cadavers that had been at 4 C for 42 and 56 d, the developmental stages of azygospores from five cadavers from each isolate were quantified.

To compare nondormancy of isolates, we conducted bioassays similar to those described above but differing in the following ways. For each isolate five cadavers were soaked in 15 mL water, gently macerated and the contents of cadavers were washed five times with distilled water. Five mL of the resulting suspension were added to each of three cups containing 20 mL sterile soil. Exposure larvae were placed on soil for 4 d periods at 15 C beginning 0, 14, 28, 42, 56 and 70 d after azygospores had been added to soil cups. The remaining cadavers were placed at 4 C, and sequential bioassays were repeated after 14, 28, 42, 70 and 96 d at 4 C.

Because azygospores of the Maryland isolate (ARSEF 5384) did not cause any infection bioassays were repeated

for this isolate with one of the Japanese isolates (ARSEF 7105) as a positive control. Because many azygospores of ARSEF 5384 were not mature (by 42 d at 4 C: C4 + C5 for ARSEF 5384 = $41.2 \pm 10.3\%$; all other isolates $\geq 90.0\%$) in the previous trial, inoculum production procedures were altered and cadavers were placed at a higher temperature during azygospore maturation. When producing azygospores, injected larvae were maintained at 25 C with 14:10 L:D until death (at 6 d) and then transferred to individual cups of 1.0% water agar at 15 C for 19 d. After 20 d the developmental stages of azygospores were quantified. Slurries of five cadavers were divided among three soil cups at 15 C as described above. Exposure larvae were maintained in soil cups for 4 d periods, beginning 14, 28, 42, 56, 70 and 84 d after azygospores were added. Cadavers that were not used were placed at 4 C, and bioassays were repeated after 14 and 28 d at 4 C.

Data analysis.—Bioassays with ARSEF 6162 comparing percent infection through time were analyzed with a logistic regression model with a binomial distribution and logit as the link function to test the effect of 4 C exposure (Proc Genmod, SAS Institute Inc, Cary, North Carolina). For sequential bioassays with ARSEF 6162 azygospores exposed to 4 C for >2 mo, treatment with mercuric chloride did not significantly increase the numbers of samples (azygospores from individual cadavers) causing larval infection of >10% of the total insects exposed ($\chi^2 = 1.6154$; $p = 0.2037$). Therefore to evaluate infection over time for samples exposed to ≥ 1 mo at 4 C, we merged the data for samples treated with or without mercuric chloride. In addition pairs of months of cold treatment were merged into four groups (1–2, 3–4, 5–6, 7–8 mo) based on similar patterns of infection and to increase group sizes to 3–6 cadavers per group. For bioassays comparing activity of fungal isolates and cold exposure, analyses were conducted without ARSEF 5384 because of the lack of infection associated with this isolate. A mixed model tested isolate and time at 4 C, with the sequential bioassays for individual azygospore samples as repeated measures (Proc Mixed, SAS Institute Inc, Cary, North Carolina). To analyze resting spore development among the six isolates for samples exposed to 4 C for 42 and 56 d, a logistic regression model (Proc Genmod, SAS Institute Inc, Cary, North Carolina) compared proportions of azygospores at C4 + C5 stages using a binomial distribution and a logit link function. Multiple comparisons of isolates after the overall tests used least squares means. For the follow-up study comparing azygospore morphologies of ARSEF 5384 and 7105 after maturation arcsine square root-transformed percentages were compared with a general linear model.

RESULTS

Azygospore germination.—Production of germ conidia was seen first 4 d after azygospores were placed on water agar and percent germination increased slowly through 12–14 d, at which time the study was discontinued because quantification of activity on plates was no longer accurate due to abundance of

germ tubes. The goal of this study was to confirm that azygospores indeed were germinating to produce germ conidia without the typical constitutive dormancy, and we recorded germ conidial production by azygospores from nine of 11 cadavers (81.8% of cadavers) by day 14, with $11.4 \pm 3.0\%$ (mean \pm SE) germination (range 0.0–26.0%) by day 12.

Nondormant azygospores with and without cold exposure.—For azygospore samples not exposed to 4 C, the first infections occurred among larvae exposed 24–28 d after resting spore had matured (FIG. 2A). By the 36–40 d bioassay, exposure larvae in all three bioassay cups were infected with $\geq 90\%$ infection from two of the samples. For exposures 42–52 d after maturation, $>90\%$ infection occurred in all cups, while all exposed insects became infected 54–58 d after exposure. After this period infection declined to $<20\%$ until 126–172 d after maturation when infection once again increased to $\geq 30\%$ of the exposed larvae. Infections occurred 24–196 d after resting spore maturation. Exposures were discontinued on day 196, although infections ($3.3 \pm 3.3\%$ infection, mean \pm SE) were still occurring. Among the 289 exposed larvae dying from *E. maimaiga* infections (total $n = 982$ larvae), only one cadaver of an exposed larva contained only azygospores (during the 66–70 d exposure), four produced both conidia and azygospores, while all others produced only conidia.

Azygospore samples exposed to cold began causing infections earlier than the samples without 4 C exposure. First infections were seen among larvae exposed 12–16 d after bioassays began for azygospores at 4 C for 1, 2, 4 and 5 mo. For azygospore samples stored in the cold 6–8 mo, first infections were seen in exposures 6–10 d after bioassays began (FIG. 2B–E).

For the 48 individual samples of azygospores (=individual cadavers) >340 naïve larvae were exposed to each sample during sequential exposures. In 26 of the 48 samples of azygospores (54.2% of the samples) $<10\%$ of the total exposure larvae became infected. For 14 of these (29.2% of the samples) a total of only 0–0.3% infection occurred among exposure larvae (0–1 exposure larvae were infected), suggesting that almost all azygospores from these cadavers were dormant. There was no clear association among the mercuric chloride treatment or the length of cold treatment and the absence of azygospore activity. To investigate patterns of infection through time we excluded samples with $<10\%$ total infection and merged samples with differing months of 4 C exposure to yield groups with 3–6 samples (FIG. 2). No pattern in infection levels among cold treatments (including no 4 C exposure) was seen

for the first 16 assays through time (≤ 94 d) for each group ($F_{4,16} = 1.92$; $p = 0.1564$) (FIG. 3A). However for the next 17 assays (>96 d) fewer exposed larvae were infected for samples with longer periods of cold exposure ($F_{4,16} = 4.08$; $p = 0.0181$) (FIG. 3B).

Azygospore samples without cold exposure demonstrated the most consistent activity (FIG. 2A), with infection occurring in a total of 65% of bioassays (including different samples across sequential exposures; TABLE II). In comparison, for samples with differing lengths of 4 C exposure, only 17–41% of bioassays resulted in infection. The associations between months at 4 C and percent of assays yielding $>0\%$, $\geq 30\%$, $\geq 60\%$, or $\geq 90\%$ infection declined through time (all r^2 s ≥ 0.332) (TABLE II). The only treatments yielding 100% infection were the azygospores without cold treatment but this level of infection only occurred soon after infections began (FIG. 2A).

For azygospore samples exposed to 4 C the same trend was seen in the types of spores formed as with samples without 4 C exposure; in almost all infected exposure larvae only conidia were produced.

Effect of origin of fungal isolates on nondormant azygospore activity.—When comparing the three North American with the three Japanese isolates, significantly fewer azygospores formed by ARSEF 5384 were C4 and C5 compared with the five other isolates ($F_{5,54} = 25.94$; $p < 0.001$) (FIG. 4). No infections occurred among larvae exposed to azygospores of this isolate. The mixed model testing isolate (minus ARSEF 5384), length of cold exposure and sequential bioassay time demonstrated no interaction between isolate and cold exposure ($F_{20,406} = 1.04$; $p = 0.4086$). Interactions between isolate*sequential bioassay time and treatment*sequential bioassay time however were significant (F tests; $p < 0.05$) but post hoc comparisons demonstrated no clear pattern in relationships. Our major question in conducting this set of studies was whether azygospores of the Japanese isolates reacted differently than North American isolates, and these studies demonstrated no clear difference other than the lack of infection from one North American isolate.

In the follow-up trial investigating the lack of infection by ARSEF 5384, fewer azygospores produced by ARSEF 5384 had reached C4 and C5 stages by 19 d ($76.4 \pm 7.7\%$), than for ARSEF 7105 ($96.6 \pm 3.2\%$), the positive control ($F_{1,8} = 8.01$; $p < 0.0222$). Once again larvae exposed to azygospores of ARSEF 5384 did not become infected while the positive control yielded ample infections. Thus, although more than half of the azygospores of ARSEF 5384 were C4 and C5, no infection occurred, suggesting that visual

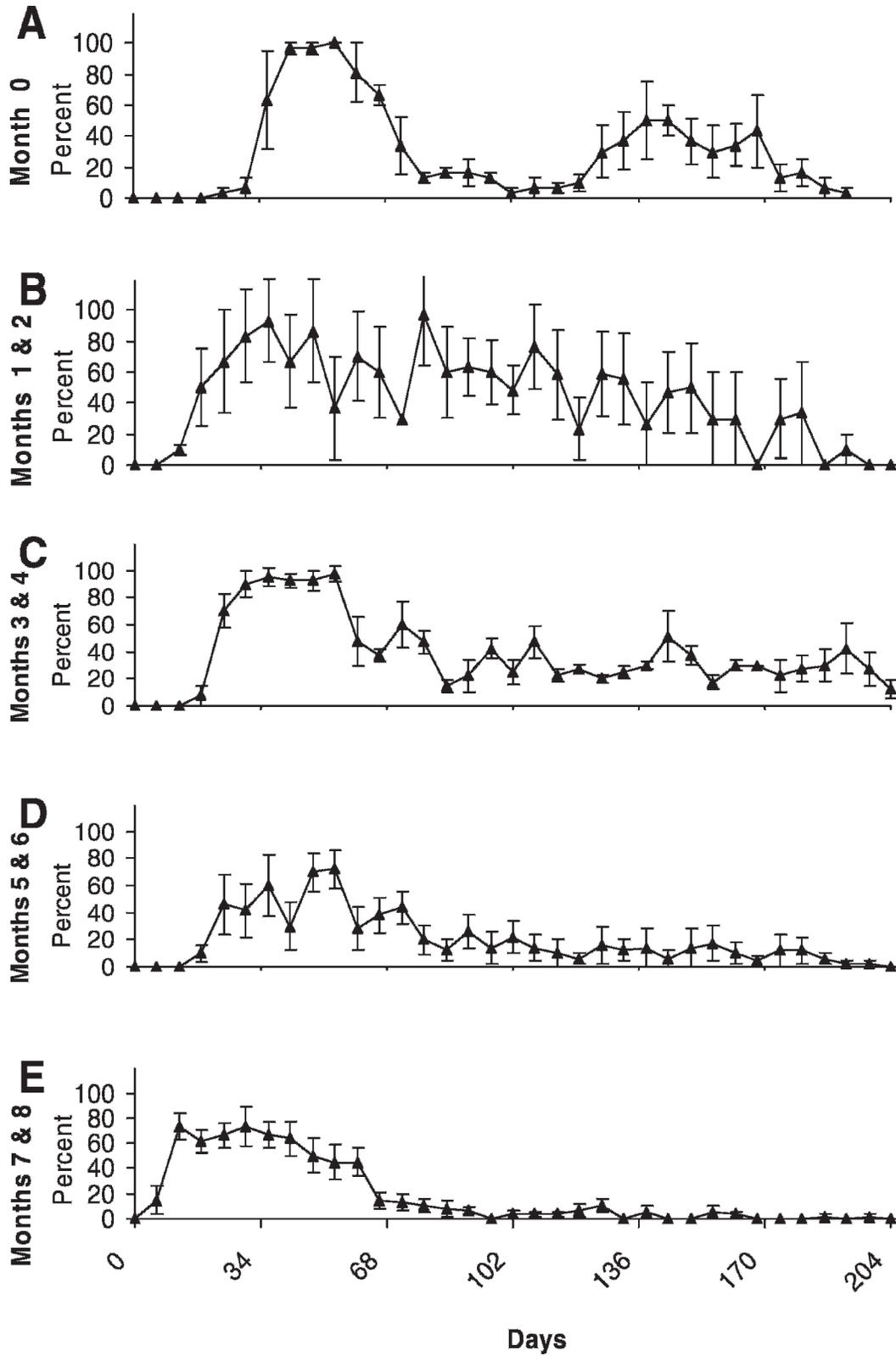


FIG. 2. Infection ($\% \pm$ SE) among groups of fourth instar gypsy moth, *Lymantria dispar*, larvae sequentially exposed to azygospores of *Entomophaga maimaiga* with no cold exposure (A) and with increasing months of cold exposure (B–E). Samples included in graphs yielded $>10\%$ total infection. Data points on the x-axis represent the timing of the first day of 4 d exposure. Months are merged based on similar patterns of infection and to increase sizes of groups.

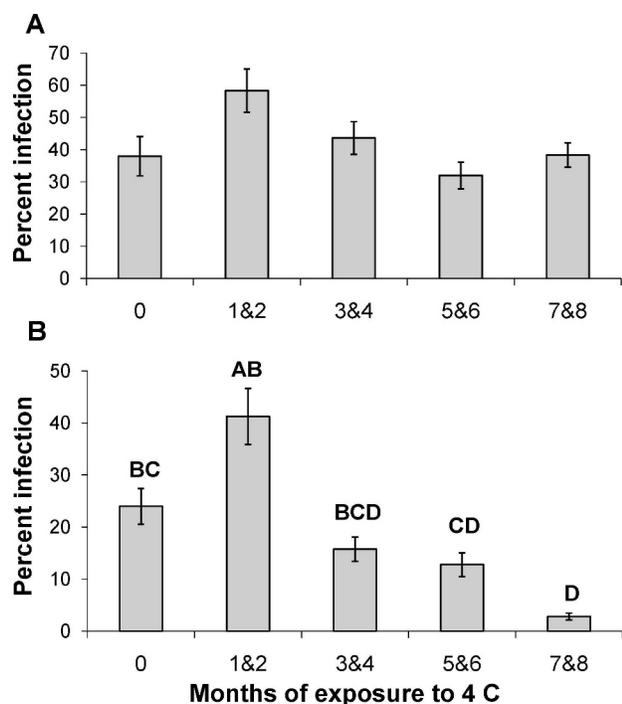


FIG. 3. Infection ($\% \pm$ SE) among groups of fourth instar gypsy moth, *Lymantria dispar*, larvae sequentially exposed to azygospores of *Entomophaga maimaiga* maintained at 4 C for variable lengths of time. Results are from sequential bioassays conducted A. ≤ 94 d or B. > 96 d after bioassay initiation. Infection at ≤ 94 d did not differ significantly by months of 4 C exposure but are presented for comparison.

evaluation of azygospore development alone (at 200 \times) does not indicate dormant state.

DISCUSSION

This is the first report of nondormancy in entomophthoralean azygospores that naturally enter constitutive dormancy. The majority of germination studies of entomophthoralean resting spores have investigated requirements for breaking dormancy and post-dormancy germination (Hajek 1997a) or germination by species that do not require dormancy (Soper et al 1975). In contrast, in the present study, we investigated germination of nondormant azygospores of *E. maimaiga*, a species with azygospores that naturally undergo dormancy. After *E. maimaiga* azygospores undergo dormancy in the field (Jul–Apr/May) they germinate asynchronously ca. 1.5–2 mo in spring. However not all spores germinate in the year after they are formed (Hajek and Humber 1997, Hajek et al 2001). In this study we demonstrated that at least some *E. maimaiga* azygospores that matured on 1.0% water agar at 20 C for 2 wk after host death did not enter constitutive dormancy but

instead began producing infective germ conidia approximately 1 mo later. Azygospores maintained in the cold after the maturation period required less than a month before infections began.

Zygospores or azygospores for the majority of species in the order Entomophthorales enter constitutive dormancy after their production, although resting spores of at least one species are not known to require dormancy. We know of no other studies where constitutive dormancy of these resting spores has been circumvented. Our laboratory is investigating the conditions required to prevent constitutive dormancy in *E. maimaiga* azygospores.

While in nature *E. maimaiga* azygospores germinate during 1.5–2 mo each year, nondormant azygospores continued infecting larvae in the laboratory 196–208 d (> 6.5 mo). Exposing nondormant azygospores to 4 C for > 5 mo yielded declining infection after 96 d, compared with results after ≤ 94 d for nondormant azygospores not exposed to 4 C or exposed to 4 C for ≤ 5 mo. We hypothesize that some nondormant azygospores could have entered dormancy at some time during or after the extended cold exposure while others remained germinable. We hypothesize that *E. maimaiga* azygospores can shift between being germinable and dormant. It is likely that in the field each spring some germinable azygospores do not fully receive the necessary conditions or stimuli to germinate and that these re-enter dormancy. This latter phenomenon of “secondary dormancy” is known from other organisms characterized by discrete germination windows for otherwise dormant propagules, for example seeds of some higher plants (Baskin and Baskin 1998), oospores of the downy mildew *Peronospora viciae* f. sp. *pisi* (phylum Oomycota, family Peronosporaceae) (van der Gaag and Frinking 1997) and oospores of the stonewort *Nitella furcata* (phylum Chlorophyta, family Characeae) (Sokol and Stross 1986). This hypothesis of occurrence of “secondary dormancy” in an entomophthoralean species must be investigated further for confirmation.

When gypsy moth larvae become infected by conidia ejected from cadavers, conidia are the predominant spore type produced from cadavers of early instars while azygospores are the predominant spore type produced in cadavers of later instars (4th–6th instars) (Hajek and Shimazu 1996). Germinating *E. maimaiga* azygospores actively eject an infective germ conidium, and when germ conidia infect larvae conidia are produced from resulting gypsy moth cadavers regardless of which larval instar was infected (Hajek 1997b). Therefore results from the present study were consistent with infections initiated by germ conidia because in almost all cases conidia were

TABLE II. Percentages of 4 d exposure bioassays demonstrating differing infection levels among samples of *E. maimaiga* azygospores exposed to 4 C for 0–8 mo

Months at 4 C	N ²	Percentages of bioassays ¹				
		0% infection	>0% infection	≥30% infection	≥60% infection	≥90% infection
0	196	35%	65%	38%	27%	13%
1	208	59%	41%	30%	7%	7%
2	208	75%	25%	14%	11%	10%
3	208	74%	26%	18%	8%	2%
4	208	83%	17%	9%	6%	5%
5	208	80%	20%	9%	6%	4%
6	208	62%	38%	19%	7%	2%
7	208	77%	23%	13%	6%	0%
8	208	80%	20%	14%	9%	6%

¹ Data across sequential bioassays and samples from each bioassay interval are pooled.

² N = total number of days after the 2 wk period for azygospore maturation that samples were bioassayed using gypsy moth larvae, with new sets of larvae exposed to azygospores for successive 4 d periods.

formed from cadavers of exposure larvae that were infected as fourth instars.

The resting spore stage has been considered a suitable stage for pest control due to its environmental resistance (e.g. Wilding et al 1986, Pell et al 2001). One major problem however has been the constitutive dormancy of resting spores after production; if constitutively dormant resting spores would be released for control it would be a long time until germination. Results from this study suggest that it is possible to produce azygospores of *E. maimaiga* that are not dormant. In addition, for nondormant azygospores stored in the cold at least 8 mo, some infections still occurred, although activity declined with increasing time in cold. Further studies are needed to understand more fully the conditions

associated with the nondormant state and conditions required for maintaining this state.

Studies comparing the abilities of azygospores of Japanese versus North American isolates to be nondormant demonstrated that this phenomenon can occur in isolates from both regions. However azygospores of one North American isolate (ARSEF 5384) would not germinate after treatment that normally resulted in nondormancy for other isolates. Our results with ARSEF 5384 suggested that differences in azygospore morphology were an indication that development of this isolate was different from other isolates. While the C4 and C5 forms of azygospores were present in this isolate but in lower titers, bioassays did not result in infections. In fact when we collected late instar gypsy moth cadavers

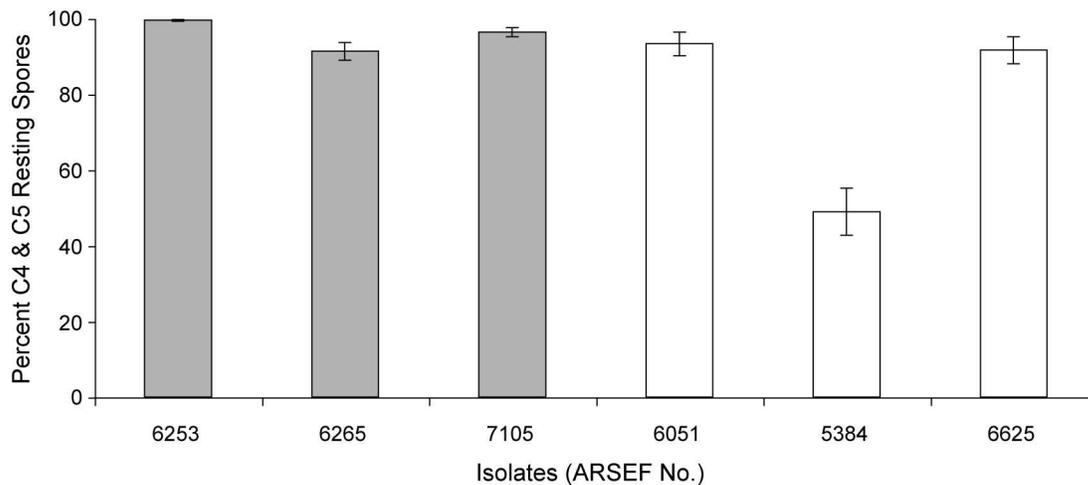


FIG. 4. *Entomophaga maimaiga* azygospores in the C4 and C5 stages (% ± SE) among three isolates from Japan (shaded bars) and three isolates from North America (open bars). Azygospores had matured in cadavers on water agar 13 d and were stored at 4 C for 42 or 56 d.

from tree trunks in the field they frequently were filled with azygospores that are C4 and C5, but these spores are dormant. Thus unfortunately C4–C5 morphology of azygospores provides only limited information about the dormancy status of azygospores. We suggest that ARSEF 5384 should be investigated further to understand whether the nondormant state can be produced in this isolate under other conditions and whether specific azygospore morphologies are in any way associated with the nondormant state.

Our study design with sequential bioassays was possible because azygospores of *E. maimaiga* are not activated by exposure to host-associated factors (Hajek and Eastburn 2001). However one potential problem with the sequential bioassay design in this study was that infective germ conidia that are actively ejected from azygospores could have been produced during a previous exposure period and remained viable on the soil. To our knowledge no studies have been conducted investigating germ conidial survival. However germ conidia morphologically resemble conidia ejected from cadavers (=primary conidia); they are the same pear shape and size is similar (ARSEF 6162 germ conidia: $29.3\text{--}30.7 \times 39.3\text{--}40.1 \mu\text{m}$ (AEH unpubl) versus ARSEF 1400 conidia: $20.6\text{--}27.6 \times 26.6\text{--}37.4$ (Soper et al 1988)). We found that when conidia of *E. maimaiga* were ejected onto sterile soil and maintained at 15 C, gypsy moth larvae became infected when exposed 14 d after conidial deposition but not 21 d (AEH unpubl). If germ conidia can also survive for some time at 15 C, then the germ conidia produced before larvae were placed in soil cups with azygospores could have caused some of the infections that were documented. It is indeed unfortunate that we were unable to conduct germination studies using azygospores in Petri dishes. However *E. maimaiga* azygospores germinate slowly (Hajek and Humber 1997) and saprophytes can overgrow plates before azygospores germinate. While antibiotics (e.g. mercuric chloride) can be used to prevent saprophytic growth these also can negatively affect azygospore germination (e.g. Hajek et al 2001). Therefore bioassays provided a much more reliable method for replication of the treatments in this study.

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