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Short Communication

Variability in azygospore production among *Entomophaga maimaiga* isolates

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ABSTRACT

This study describes *in vitro* and *in vivo* azygospore production by nine isolates of *Entomophaga maimaiga*, a fungal pathogen of the gypsy moth, *Lymantria dispar*. The three *E. maimaiga* isolates that consistently produced azygospores *in vitro* were also strong producers of azygospores *in vivo*. However, two additional isolates that were strong azygospore producers *in vivo* did not produce azygospores *in vitro*. Isolates that produced azygospores *in vitro* produced both conidia and azygospores more frequently *in vivo* than isolates not producing azygospores *in vitro*. *In vitro* azygospore production varied over time as well as by isolate. After >2 years of cold storage, while three isolates continued *in vitro* azygospore production, three isolates no longer produced azygospores *in vitro*.

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1. Introduction

Species of entomophthoralean fungi are well known for their ability to cause epizootics but difficulties in mass production have hindered their practical application for pest control (Pell et al., 2001). Most species of Entomophthorales make two major types of spores, asexually produced conidia, that are ready to germinate and infect after production, and long-lived azygospores or zygospores, commonly called resting spores, that are usually environmentally persistent; these two spore types play significantly different roles in different stages of the fungal life cycle. For the gypsy moth (*Lymantria dispar*) larval pathogen *Entomophaga maimaiga*, either one type of spore or both are produced after an infected host has died. *E. maimaiga* resting spores have been suggested as the best stage for release to help control gypsy moth populations (Hajek, 1999). The type of spore produced *in vivo* by *E. maimaiga* is influenced by host instar as well as isolate, serial passage, inoculum density, temperature and moisture (Hajek and Shimazu, 1996). Our laboratory has investigated production and use of *E. maimaiga* azygospores for control of the gypsy moth. We documented *in vitro* production of *E. maimaiga* azygospores in cell culture media and found this was strongly associated with specific fungal isolates (Kogan and Hajek, 2000).

Papierok et al. (1984) stated that finding the right isolate was very important for developing species of Entomophthorales for biological control. Yet, few studies have been conducted comparing sporulation across enthomophthoralean isolates. Isolates of the entomophthoralean aphid pathogen *Conidiobolus obscurus* (= *Entomophthora obscura*) have been grouped into three categories: isolates capable of forming azygospores both *in vivo* and *in vitro*, isolates capable of forming azygospores only *in vivo* and isolates that never form azygospores (Latgé et al., 1979). We investigated whether *E. maimaiga* isolates producing azygospores *in vitro* also produced azygospores and conidia *in vivo*. We also show that *in vivo* production of *E. maimaiga* azygospores can be transient in some isolates.

2. Materials and methods

Gypsy moth larvae used for studies were obtained as neonates from the USDA, APHIS, Otis Methods Development Laboratory and were reared at 23 °C, 65% humidity on high wheat germ artificial diet (Bernon, 1995). Cultures of *E. maimaiga* used in this study were initially obtained from the USDA, Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF) in Ithaca, New York. Nine *E. maimaiga* isolates were chosen for this study, with three isolates each previously documented as producing high, medium or low titers of azygospores *in vitro* during a study conducted approximately 2.5 years prior to the present study (Kogan and Hajek, 2000) (Table 1). Isolates were stored at −80 °C in 10% glycerol until the present study. For the present study, isolates were thawed and 1 mL of the fungal inoculum was grown at 20 °C in the dark in 9.5 mL Grace's insect cell culture medium plus 0.5 mL fetal bovine serum (GIBCO, Gaithersburg, Maryland) (Kogan and Hajek, 2000). After one week, 1 mL of each isolate was transferred to new media and 2 days later the resulting protoplast suspensions were used to infect insects and inoculate culture flasks to measure *in vivo* and *in vitro* spore production. To infect larvae, the protoplast suspension was adjusted to 1×10^5 cells/mL and 10 μL were injected into each of 50 fourth

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Table 1
Isolates of *Entomophaga maimaiga* and spore production.

ARSEF number	Collection location	Collection year	Prior *in vitro* azygospore prod.[a,b]	*In vitro* azygospore prod.[a]	*In vivo* azygospore prod.[c]	% Cadavers with both azygospores and conidia[d]
5384	Sudlersville, Maryland	1994	–	–	5	39.3 ± 13.9
5391	Yellow Barn State Forest, New York	1996	+	++	4	79.9 ± 3.1
5392	Yellow Barn State Forest, New York	1996	–	–	5	93.4 ± 1.7
5394	Yellow Barn State Forest, New York	1996	++	++	4	68.9 ± 13.9
5563	Quabbin Reservoir, Massachusetts	1996	++	++	5	99.0 ± 1.0
5569	Archer Run, Virginia	1996	+	–	3	15.0 ± 5.0
5571	Elliot Spring Run, Virginia	1997	–	–	0	0.0 ± 0.0
5711	Quabbin Reservoir, Massachusetts	1996	+	–	3	29.4 ± 27.3
6168	Hawes Run, West Virginia	1994	++	-	3	10.5 ± 10.5

[a] *In vitro* azygospore production: –, no azygospores produced *in vitro*; +, 30–200 azygospores/mL; ++, >1000 azygospores/mL.
[b] As reported in Kogan and Hajek (2000) from studies conducted ca. 2.5 years before the present studies.
[c] Mean *in vivo* azygospore production. Isolates were categorized by the truncated log of the mean azygospores produced in fourth and fifth instar larvae, e.g. an isolate producing 3.6×10^5 azygospores/cadaver has a log value of 5.53 and a truncated log value of 5.
[d] Mean percentage ± SE of cadavers of fourth and fifth instar gypsy moth larvae producing both conidia and azygospores.

and 50 fifth instar larvae at the base of a proleg. After injection, gypsy moth larvae were placed into 29.6 mL cups of artificial diet at 20 °C, 14:10 (L:D) and were monitored on days 6–8 after injection for death. On the day that each larva died, its cadaver was placed individually into a 29.6 mL plastic cup containing 1.5% sterile water agar. Over the next 4 days, conidial production by each cadaver was rated as none, weak (<75% of the cadaver covered with conidiophores) or strong (⩾75% of the cadaver covered with conidiophores). For each conidiation level/instar group, *in vivo* resting spore production was quantified for a maximum of 10 cadavers (mean ± SE: 6.4 ± 0.6 cadavers per conidiation level/instar group). Each of the cadavers to be quantified was soaked in 2 mL distilled water for 30 min, macerated with a metal spatula for 30 s and the suspension was then vortexed for 1.5 min. Resulting suspensions were quantified using a hemocytometer to calculate azygospores/mL, which was multiplied by the cadaver suspension volume (cadaver volume plus 2 mL) to determine azygospores/cadaver.

To evaluate *in vitro* growth, 0.25 mL of the 1×10^5 protoplasts/mL suspension used for injection was added to 1.9 mL Grace's insect cell culture medium plus 0.1 mL fetal bovine serum in a 25 cm^2 cell culture flask (Corning, New York), with five replicate flasks per isolate. Cultures were grown at 20 °C in the dark. On day 21, azygospores in each flask were counted in three 0.3 cm^2 areas within each flask, using an inverted Olympus IX50 microscope at 200× magnification (Kogan and Hajek, 2000). Counts per flask were averaged and converted to azygospores/mL based on the total volume per flask.

In vivo resting spore production per cadaver was compared across conidiation levels using a zero-inflated negative binomial analysis (STATA, 2007), due to the large number of samples with no azygospores.

3. Results and discussion

Three isolates (ARSEF 5391, 5394 and 5563) produced from 1280 to 7000 azygospores/mL *in vitro*; these isolates had previously produced azygospores *in vitro* (Table 1). Two of these isolates (ARSEF 5394 and 5563) maintained the high *in vitro* production reported in Kogan and Hajek (2000) while the only other isolate producing *in vitro* azygospores (ARSEF 5391) in this study, increased from 30 to 200 azygospores/mL recorded in Kogan and Hajek (2000) to $1.32 \times 10^3 \pm 6.42 \times 10^2$ azygospores/mL in this study. Three isolates that had previously produced azygospores *in vitro* did not do so in this study (ARSEF 5569, 5711 and 6168). The three isolates that previously had not produced any azygospores *in vitro* continued not to produce azygospores *in vitro* (ARSEF 5384, 5392 and 5571) (Table 1). In a study of the effects of cryopreservation on three isolates of *E. maimaiga*, there was a trend of decreased azygospore production *in vivo* after storage of cultures under liquid nitrogen for 7 years (Hajek et al., 1995). While for the present study, the contribution of cryopreservation to changes in azygospore production *in vitro* is not known, it is clear that azygospore production by different isolates can change through time.

One isolate (ARSEF 5571) made no azygospores in either fourth or fifth instars, while two additional isolates (ARSEF 5569 and 6168) did not produce azygospores in fourth instars and fifth instars, respectively; none of these isolates made azygospores *in vitro*. For those isolates producing azygospores *in vivo*, the average per cadaver was $1.32 \times 10^5 \pm 5.00 \times 10^4$ azygospores for larvae infected as fourth instars and $2.00 \times 10^5 \pm 7.94 \times 10^4$ for larvae infected as fifth instars. The density of azygospores/cadaver in fourth instars was positively associated with the density of azygospores/cadaver in fifth instars ($r = 0.848$; $P = 0.016$; PROC CORR) (SAS Institute, 2004).

We had hypothesized a trade-off between azygospore and conidial formation *in vivo*. However, isolates that did not produce conidia *in vivo* produced significantly fewer azygospores *in vivo* than isolates producing conidia *in vivo* ($z = -2.50$, $P = 0.012$ for no conidia vs. weak conidia production, $z = -2.07$, $P = 0.038$ for no conidia vs. strong conidial production; Fig. 1). Further, among isolates that produced both conidia and azygospores *in vivo*, there was no significant trade-off between the amounts of each spore type produced ($z = -0.85$, $P = 0.397$; Fig. 1).

When we compared *in vitro* and *in vivo* spore production, we found that the three isolates that made azygospores *in vitro* were also among the strongest isolates for *in vivo* spore production. For the three isolates that consistently produced azygospores *in vitro* (ARSEF 5391, 5394 and 5563), all produced >10^4 azygospores/cadaver and both conidia and azygospores were produced in ⩾65% of fourth and fifth instar cadavers (Table 1). In contrast, for isolates not producing azygospores *in vitro*, while two produced >10^4 azygospores/cadaver, only one (ARSEF 5392) produced both spore types in ⩾50% of cadavers. Isolates that produced azygospores *in vitro* produced both spore types in a significantly higher percentage of cadavers (82.6 ± 8.8%) than isolates that did not

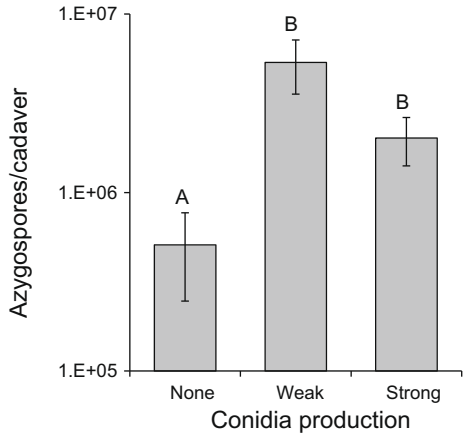

Fig. 1. Azgospore production (mean ± SE) in cadavers that produced different amounts of conidia. Conidial production by cadavers was rated as none, weak (<75% of the cadaver covered with conidiophores) or strong (⩾75% of the cadaver covered with conidiophores).

produce azygospores *in vitro* (31.3 ± 13.7%) ($\chi^2 = 234.27$, $P < 0.0001$). However, there was no difference in the percentages of cadavers with strong conidial production for isolates making azygospores *in vitro* or not ($\chi^2 = 0.40$, $P = 0.5262$). Therefore, we could not identify characteristics of *in vivo* spore production that helped to predict *in vitro* azygospore production, aside from a general trend of strong azygospore production *in vivo* associated with production of both spore types in high percentages of late instar cadavers.

Few studies have been conducted of variation by isolate in types of spores produced for species of Entomophthorales that produce azygospores and conidia. In studies comparing production of azygospores *in vivo* by different *E. maimaiga* isolates, 21 of 25 isolates produced azygospores in later instar gypsy moth (Hajek and Shimazu, 1996; Nielsen et al., 2005; Hajek et al., 2008; M. Bertoia, personal communication; M. Shimazu, personal communication). In contrast, Latgé et al. (1979) stated that most *C. obscurus* isolates did not produce azygospores *in vitro*. For *in vitro* culture, Latgé and Boucias (1984) reported that 6 of 25 isolates (24%) of *C. obscurus* made azygospores in both liquid and solid culture, nine isolates (36%) only made azygospores in solid culture and 10 isolates (40%) made no azygospores *in vitro*; isozyme analysis of these isolates grouped isolates associated with their ability to produce azygospores *in vitro*. While azygospore production by *E. maimaiga* in solid media has never been reported, Kogan and Hajek (2000) demonstrated that 74% of 38 *E. maimaiga* isolates produced azygospores in liquid culture (cell culture media). However, in the present study, only 3 of 9 isolates (33.3%) produced azygospores in liquid culture, although 8 of the same 9 isolates produced azygospores *in vivo*; surprisingly three of the nine had previously made azygospores *in vitro* prior to cold storage. Thus, *in vitro* production of azygospores by both *E. maimaiga* and *C. obscurus* can vary by isolate both *in vivo* and *in vitro* and the ability to produce azygospores by *E. maimaiga* isolates can change over time. Differences in spore production among entomophthoralean isolates and changes in entomophthoralean spore production over time, including in association with cold storage, clearly warrant further study to improve our understanding of these processes.

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