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Distribution of resting spores of the *Lymantria dispar* pathogen *Entomophaga maimaiga* in soil and on bark

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Abstract. Cadavers of late instar *Lymantria dispar* (gypsy moth) larvae killed by the fungal pathogen *Entomophaga maimaiga* predominantly contain resting spores (azygospores). These cadavers frequently remain attached to tree trunks for several weeks before they detach and fall to the ground. Density gradient centrifugation was used to quantify resting spores in the soil and on tree bark. Titers of resting spores were extremely high at 0–10 cm from the base of the tree and the number decreased with distance from the trunk of the tree. Titers were also highest in the organic layer of the soil with numbers decreasing precipitously with increasing depth in the soil. While resting spores were obtained from tree bark, densities per unit area were much lower than those found in the organic soil layer at the base of the tree. Field bioassays were conducted with caged *L. dispar* larvae to compare infection levels with distance from the tree trunk as well as on the trunk. Highest infection levels were found at 50 cm from the tree base with lowest infection on the tree trunk at 0.5 m height, although we expected the highest infection levels among larvae caged at the bases of trees, where highest spore titers occurred. Laboratory experiments demonstrated that *L. dispar* larvae exposed to resting spore-bearing soil at the soil surface became infected while larvae exposed to soil with resting spores buried at least 1 cm below the surface did not become infected.

Key words: azygospores, Entomophthorales, epizootiology, fungal distribution, soil extraction

Introduction

Many entomopathogenic fungi in the Entomophthorales are obligate pathogens associated with hosts that inhabit epigeal habitats. Infective spores of these fungi, both germ conidia from resting spores (zygospores or azygospores) and primary conidia produced from cadavers, are frequently forcibly discharged. Entomophthoralean conidia can therefore be abundant in the air spora (e.g. Steinkraus et al., 1996). It has generally been considered that the non-infective, environmentally resistant resting spores of Entomophthorales, although formed in cadavers above the soil, typically fall

to the ground and accumulate in the soil. Other entomopathogenic fungi that are known to occur in the soil, e.g. *Beauveria bassiana* and *Metarhizium anisopliae*, are frequently associated with soil-dwelling hosts (Keller and Zimmermann, 1989). Soil has been confirmed as a reservoir for resting spores for the entomophthoralean species *Conidiobolus obscurus* (MacDonald and Spokes, 1981) and *Entomophaga maimaiga* (Hajek and Wheeler, 1994). Curiously, hosts for these entomophthoralean fungi, respectively aphids and *Lymantria dispar* larvae, do not have soil-dwelling stages. These epigeal dwellers can become infected when in contact with the soil surface, as shown when aphids (Latteur, 1977) and *L. dispar* larvae (Hajek and Humber, 1997) have been caged on the soil surface. Studies are presently being conducted to investigate when and where *L. dispar* larvae that feed on foliage of forest trees become infected by germ conidia from soil-borne resting spores. While we know that resting spores initiate the cycles of secondary infection that can result in epizootics, the distribution of this pathogen reservoir, e.g. entomophthoralean resting spores, in the environment has never been evaluated.

The entomophthoralean pathogen of *L. dispar*, *E. maimaiga*, was first detected in North America in 1989 (Andreadis and Weseloh, 1990; Hajek et al., 1990). Resting spores (azygospores) of *E. maimaiga* are predominantly produced in cadavers of late instars dying from *E. maimaiga* infections (Hajek and Shimazu, 1996). These environmentally resistant spores range between 21.7–40.3 μm in diameter (Soper et al., 1988). Late instar *L. dispar* cadavers often hang on tree trunks for several weeks after host death, then fall to the soil and resting spores are subsequently leached from decomposing cadavers into the soil (Hajek et al., 1998). Densities of *E. maimaiga* resting spores can be exceptionally high immediately surrounding the bases of trees, the only place previously sampled for quantification of *E. maimaiga* resting spores (Hajek and Wheeler, 1994). We present data on densities of resting spores at varying distances from bases of tree trunks and at varying depths in the soil. Because resting spores have previously been obtained from tree bark (Hajek, unpublished data) and larvae in association with bark have become infected by *E. maimaiga* (Shimazu et al., 1987; Hajek and Roberts, 1991), densities of resting spores on tree bark were also quantified. Bioassays were conducted to investigate the prevalence of infections among larvae exposed to the soil surface at varying distances from trunks of trees or on the bark surface of tree trunks. During laboratory studies, we also evaluated the ability of resting spores that occur at varying soil depths to infect larvae.

Materials and methods

Study site

Soil was collected 30 November and 1 December 1995 in Queen Anne's County on the eastern shore of Maryland. The site was completely forested with mixed hardwoods and the soil for this area is Woodstown sandy loam with 0–2% slope. *L. dispar* populations in the collection site had been sampled the previous May and June and high levels of *E. maimaiga* infection had been documented. On three of the four trees sampled, a few *L. dispar* cadavers containing *E. maimaiga* resting spores were still partially present on the tree bark on the soil collection dates.

Soil collection protocol

Soil was sampled from the four cardinal directions around the bases of four red oak (*Quercus rubra* L.) trees. The average canopy width from the four trees was 3.9 m (SE = 0.6; range = 3.3–5.8 m). In each direction, samples were taken 0–10, 50 and 100 cm from the base of the tree. For each tree, in one direction that was randomly chosen by tree, samples were also taken at 200 cm from the tree base. For one tree, samples were taken at 300 cm only toward the south. For each collection location, samples were taken at a variety of depths. First, loose leaves were brushed from the surface. Then, a sample was taken from the dark, high organic content layer containing roots (O_e horizon; Kohnke and Franzmeier, 1995). To take this sample a trowel was used to remove this layer over a 5 × 5 cm area. Subsequent samples at each location were taken in the mineral soil. A standard soil corer was used to extract cores from 0–3 cm, 3–8 cm, and 8–13 cm. For transects including samples taken at 200 cm from the tree base, additional samples were taken at the depth of 13–18 cm. Extreme care was taken not to contaminate lower samples with soil from above. Samples from 0–3 cm were usually the A horizon, 3–8 cm were a mixture of A and B horizons, and 8–13 cm and lower were usually the B horizon. Not all locations could always be sampled at all depths; for the sample 0–10 cm from the base of the tree, usually only the 0–3 cm depth could be sampled due to the presence of roots. Samples were immediately placed in double plastic freezer bags and were stored at 4 °C.

All samples were weighed, dried at 60 °C overnight, and weighed once more to determine moisture level. Resting spores were quantified using Percoll density gradient centrifugation (Hajek and Wheeler, 1994). Briefly, a 5 g subsample of soil from a location was placed in a dilute detergent solution and sonicated for 1 min. The sample was then wet-sieved, with resting spores collected on a 20 μm sieve. Discontinuous density gradients from 1.13 g/ml to 1.05 g/ml were created and sieved material was layered on top. Gradients were centrifuged at 4000 g for 10 min and any resting spores in the

samples were counted at 50× on an Olympus SZH dissecting microscope. Resting spores/g of dry soil was calculated using accuracy conversions developed specifically for this technique (Hajek and Wheeler, 1994). For statistical analysis, resting spore counts (including bark counts described below) were transformed using (log(x) + 1) to stabilize the variance.

Bark collection protocol

Bark samples were taken in the four cardinal directions at 0.5, 1.0, 1.5, and 2.0 m above the ground. Each sample consisted of a 5 × 5 cm square of bark, cut with a chisel down to below the deepest crevice. Care was taken to collect all pieces as bark squares were cut. Bark samples were placed in double plastic bags and stored at 4 °C.

Field bioassays

Bioassays were conducted using three of the four trees from which soil and bark samples were taken. *L. dispar* larvae were caged at varying distances from the trunks of the trees using 46 × 15.5 cm pieces of standard window screening folded in half (23 × 31 cm) and stapled around the edges. Twenty-five fourth instar larvae were placed within each cage and the open sides were stapled closed. Leaf litter was brushed aside and cages were placed on the surface of the O_e layer of the soil at the tree base and at 50, 100, and 200 cm from the trunk for a period of twelve hours beginning at 0800. Throughout the exposure period, cages were covered with plastic boxes (32 × 17 × 9 cm) to prevent larvae from being exposed to airborne conidia settling onto cages. These experiments were conducted four times between 29 May and 6 June 1996. During this period, the resident *L. dispar* population was predominantly fourth and fifth instars. After their exposure, larvae were placed on high wheat germ artificial diet (Bell et al., 1981) in 237 ml plastic cups in groups of 10 at 20 °C. Larvae were checked daily for mortality and any resulting cadavers were evaluated for cause of death as described previously (Hajek and Humber, 1997). Because larvae were reared in groups, only data for larval mortality during the first five days after exposures were included; this was necessary in order to avoid inclusion of data from secondary infections that might have occurred within diet cups. Percent infection was arcsine transformed to conduct multiple t-tests partitioning the α of 0.05.

Soil depth bioassays

The soil used for these studies was collected at the George Washington National Forest, Deerfield Ranger District, Virginia, USA on 8 April 1997. Only the organic layer of soil (O_e) within 10 cm of oak trunks was collected. Using wet sieving and density gradient centrifugation, the density of *E. maimaiga* resting spores was estimated at 6743 ± 352 resting spores/g dry

soil. The soil was maintained at 4 °C until experiments were initiated on 10 June.

Experiments were conducted in 0.95 liter plastic freezer containers (9.5 × 9.5 × 12.0 cm) that could be sealed tightly. Resting spore-laden soil was adjusted to near saturation. Soil filled the containers within 15 mm of the lid so that larvae were in proximity to the soil at all times. For each treatment, only one centimeter of soil with spores was used per container, but the location of this resting spore-laden soil was changed. For the 0 cm treatment, 1.5% water agar filled the container to 9 cm height with one centimeter of field-collected soil on top. For the remaining treatment, resting spore-laden soil was placed under 1 cm of nearly saturated autoclaved organic-layer soil with an appropriate decrease in the level of water agar below the field-collected soil.

For each experimental replicate, 10 larvae were placed in each of three containers per treatment and maintained at 15 °C and 14:10 L:D for 4 days. During this period of time, larvae were not provided with food in order to avoid growth of saprophytes in experimental containers. After four days, larvae were removed from the soil and placed individually into 29 ml plastic cups that were one-third filled with high wheat germ artificial diet (Bell et al., 1981) and maintained at 20 °C and 14:10 L:D. Larvae were checked for mortality daily for 10 days and any cadavers were checked for the presence of conidia and resting spores as previously described (Hajek and Humber, 1997). This experimental protocol was replicated twice comparing resting spore-laden soil at 0 and 1 cm.

Results

Distribution in soil and on bark

Resting spores of *E. maimaiga* predominantly occurred at 0–10 cm from tree trunks, with progressively decreasing densities at 50 and 100 cm (*t*-test using Sidak inequality for data from O_e and 0–3 cm soil layers; $p < 0.05$). For the few samples taken at 200 and 300 cm from the tree trunk, this trend continued (Table 1).

Resting spores of *E. maimaiga* were found predominantly in the O_e layer of the soil compared with the 0–3, 3–8, and 8–13 cm layers at 50 and 100 cm (*t*-test using the Sidak inequality; $p < 0.05$) (Table 1). Because the depth of soil was limited at the bases of trees, next to the tree trunk few samples could be taken at a depth >3 cm. Therefore, a separate analysis comparing densities at O_e and 0–3 cm determined again that resting spore densities were greater in the organic layer (*t*-test using the Sidak inequality; $p < 0.05$). The few

Table 1. Densities of *E. maimaiga* resting spores by distance from tree trunks and depth in sandy loam soil. Mean resting spores per gram dry soil were calculated across four red oak trees. Results of statistical analyses comparing densities by depth or distance are presented in the text

	Distance from tree trunk									
	0–10 cm		50 cm		100 cm		200 cm		300 cm	
Soil depth	n	Mean ± SE	n	Mean ± SE	n	Mean ± SE	n	Mean ± SE	n	no.
O_e layer	16	4751 ± 1538	15	104 ± 37	15	16 ± 9	4	2 ± 2	1	11
0–3 cm	16	333 ± 37	15	23 ± 19	15	2 ± 1	4	32 ± 30	1	0
3–8 cm	2	18 ± 9	15	8 ± 3	15	12 ± 12	4	3 ± 3	1	0
8–13 cm		–	15	3 ± 1	15	1 ± 0	4	0 ± 0	1	0
13–18 cm		–	3	6 ± 3	4	7 ± 3	4	0 ± 0	1	0

Table 2. Densities of *E. maimaiga* resting spores extracted from tree bark at varying heights above ground. Mean resting spores per 25 cm^2 was calculated for four sample trees

	Resting spores/25 cm^2 bark*	
Height above ground	n	Mean ± SE
0.5 m	15	277 ± 52 a
1.0 m	16	133 ± 37 b
1.5 m	15	69 ± 26 bc
2.0 m	16	44 ± 21 c

* Means followed by differing letters are significantly different (*t*-test using Sidak inequality; $p < 0.05$).

samples evaluated for resting spore densities at the 13–18 cm depth indicated that very few spores occur at this depth in the mineral soil.

On the bark, resting spore densities were greatest at 0.5 m and densities declined with increasing height (*t*-test using Sidak inequality; $p < 0.05$) (Table 2). Interestingly, soil contaminated the bark of 50 cm height samples and it cannot be distinguished whether resting spores in these samples originated from soil which was splashed on the trunks of trees or whether resting spores were leached from cadavers when these were attached to the bark. Even with the potential for soil bearing resting spores being splashed onto tree trunks, densities of resting spores were much lower on bark than on soil. The O_e layer averaged 5.0 ± 0.5 cm (mean ± SE) thick at the bases of tree trunks to 6.4 ± 1.4 cm thick at 200 cm from the tree trunks. Resting spores

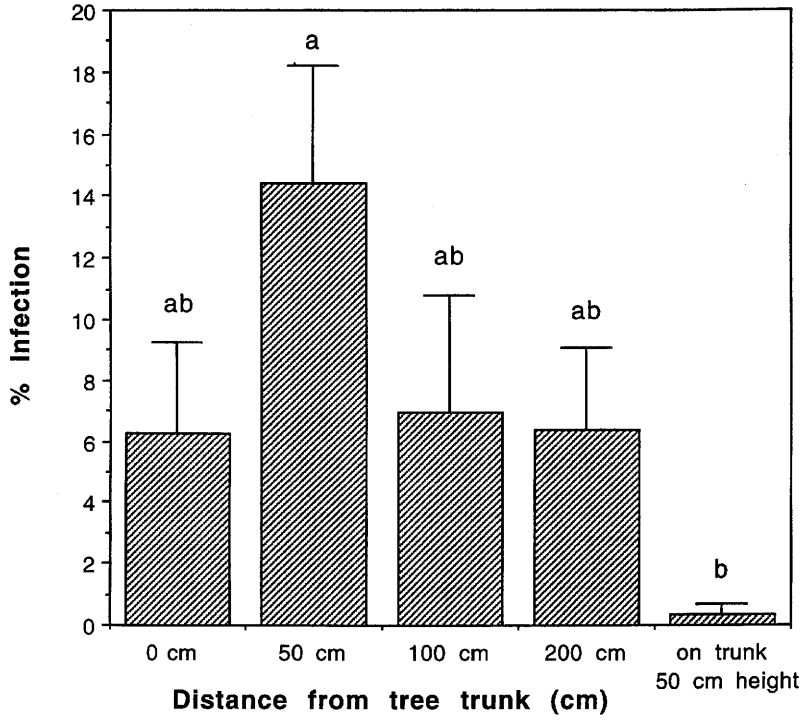

Figure 1. Percent *E. maimaiga* infection among gypsy moth (*Lymantria dispar*) larvae caged on top of the soil at differing distances from tree trunks and on the trunk 50 cm above the ground.

in soil were evaluated per gram of soil (Table 1) and from these calculations we can estimate the number of resting spores in sections of the O_e that were 25 cm^2 on the surface to compare with bark counts per 25 cm^2 areas. Based on counts from 0–10 cm around bases of the trees sampled, within a 25 cm^2 area of the O_e, an average of 62118 $\pm$ 14507 resting spores would be found. While this value is much greater than the densities of resting spores in bark samples (Table 2), direct comparisons are difficult because soil samples were three dimensional while bark samples were two dimensional; resting spores could only reside on the surface of the bark while they probably occurred throughout the soil samples. Unfortunately, for soil samples we are unable to estimate the number of resting spores on only the surface of the soil or which resting spores would be able to send germ tubes to the soil surface. Nevertheless, the average density in the O_e is over 200 times greater than the most abundant counts on bark (Table 2), suggesting that bark is not the primary overwintering location for resting spores.

Field bioassays
No differences in infection level were detected among larvae caged on the soil at different distances from tree trunks (*t*-tests using the Sidak inequality; $p > 0.05$) (Figure 1). Nevertheless, a trend was obvious with the greatest infection occurring at 50 cm. The least infection was seen among larvae caged on tree trunks with percent infection significantly different from larvae caged at 50 cm on the soil ($p < 0.05$).

Soil depth bioassays
Only *L. dispar* larvae exposed directly to resting spore-laden soil became infected (90 ± 5.8%, 63.3 ± 17.6%; means ± SE for two replicates). When resting spore-laden soil was 1–2 cm below the surface on which larvae were maintained, no larvae became infected.

Discussion

The majority of *E. maimaiga* resting spores are distributed in the soil close to the bases of trees. Late instars, in which resting spores are formed, frequently die attached to tree trunks by their prolegs, and most cadavers subsequently fall to the soil surface directly around the bases of trees; cadavers eventually disintegrate and resting spores are apparently leached into the soil at these locations (Hajek et al., 1998). Therefore, resting spores are highly aggregated in the soil around the bases of trees. The densities of resting spores in soil did not differ among trees in the small area sampled during this study. However, except in outbreak situations, gypsy moth populations are aggregated and not randomly distributed (Liebhold and Elkinton, 1989). Therefore, we assume that over a larger area, densities of resting spores would vary based on the distribution of gypsy moth larvae and how many cadavers were attached to individual tree trunks.

E. maimaiga resting spores were predominantly found in the organic layer of the soil although at the tree base where they were most dense resting spores were fairly abundant also in the upper 3 cm of mineral soil. While occasionally these relatively large spores appeared to move through the organic layer to the mineral soil, the soil appeared to act as a filter, so that especially below 3 cm resting spores were uncommon. Those spores that were found at greater depths probably moved through the soil via soil cracks or spaces created by the movement of invertebrates or decomposition of tree roots (P. Arneson, Cornell University, Ithaca, NY, USA, 1998, personal communication). In agreement with our findings, when the $\approx 2.5 \times 2.5$ μm diameter conidia of *B. bassiana* were added to soil, >94% were recovered in the upper 5 cm of soil and few conidia penetrated to depths of 5.1–15.0 cm; mechanical filtering of

conidia by the soil was suggested as the cause for this distribution (Storey and Gardner, 1988). Based on our experimental results, resting spores located at greater depths in the mineral soil would have little or no chance of infecting *L. dispar* larvae unless they were relocated to the soil surface, possibly due to soil erosion or in association with the movement of invertebrates.

Resting spores were found on the bark at very low numbers compared with the density found in the organic layer of soil at the base of the trees. After late instars die attached to tree trunks, many cadavers become desiccated and then fall to the ground without breaking. We hypothesize that resting spores are deposited on bark when cadavers rupture and release resting spores while still attached to trunks, or when resting spores are leached from attached cadavers by rain or when resting spore-laden soil is splashed onto trunks.

Infection levels among *L. dispar* larvae caged at locations from which resting spores were quantified were lowest for those larvae caged on tree trunks. These results are consistent with findings that resting spores per area are much lower on bark surfaces than in soil at the bases of trees. For larvae caged on the surface of the soil, bioassay results did not differ with distance from the tree trunk although the level of infection was greatest at 50 cm. In fact, infection level was more than two times greater 50 cm from the tree trunk compared with 0–10 cm, yet spore counts were almost 50 fold lower. Based on these resting spore counts, we assumed that infection levels would be highest for larvae caged at 0–10 cm on the soil, where resting spore densities were greatest. The trees that were used for these studies were unusual because they were devoid of leaf litter in the slightly elevated area immediately surrounding the bases of the tree trunks (at 0–10 cm), while at 50 cm, leaf litter covered the soil. We know that high resting spore activity is associated with high soil moisture (Hajek and Humber, 1997). We hypothesize that for these individual trees, the soil in the area 0–10 cm from the bases of the trees did not stay as moist as the surrounding area because it was exposed, i.e., not covered by leaf litter, and therefore resting spores in this area would not have germinated as abundantly as in the region further away from the bases of the trees that was covered by leaf litter.

While density gradient centrifugation is relatively accurate using calculated correction factors (Hajek and Wheeler, 1994), we should mention that all resting spores with similar morphology are counted using this method. Many members of the Entomophthorales have resting spores with similar to identical morphology. However, we feel that this method was used accurately in the present study because cadavers of late instar *L. dispar* killed by *E. maimaiga* were abundantly hanging on tree trunks the summer before samples were taken and no evidence of other species of Entomophthorales was detected. However, a better quantification of *E. maimaiga* resting spores

could be realized if a method was developed that differentiated between resting spores of different entomophthoralean species.

Soil is considered the primary reservoir for many species of entomopathogenic fungi and it appears this is also true for *E. maimaiga*. In this study, the soil in the area being sampled was a sandy loam covered by a thick organic layer. For the entomopathogenic fungus *Nomuraea rileyi*, studies showed that conidia washed through sand much more readily than through loam (Ignoffo et al., 1977). Further study will be necessary to compare potential differences in the retention of viable resting spores by soils having little or no organic layer and/or mostly clay or highly sandy soils.

Although soil is considered the reservoir for many entomopathogenic fungi, resting spores of entomophthoralean species associated with aphids (Byford and Ward, 1968) and a psyllid (Dustan, 1924) have been assumed as overwintering on and under bark of orchard trees. In agreement with our findings, *L. dispar* larvae exposed in spring to trunks of *Lithocarpus aedulis* trees that had been covered with cadavers bearing *E. maimaiga* resting spores the previous summer became infected (Shimazu et al., 1987). In addition, the entomopathogenic hyphomycete *Beauveria bassiana* has previously been isolated from elm bark, probably originating from cadavers of *Scolytus scolytus* (Doberski and Tribe, 1980). Whether retention of *E. maimaiga* resting spores on tree bark might vary by tree species remains to be explored.

It is possible that resting spores on tree trunks do not survive as long as soil-borne resting spores. *E. maimaiga* resting spores are now known to survive in soil at least six years after production (Weseloh and Andreadis, 1997). In contrast, experiments have shown that larvae exposed to tree bark assumed to harbor *E. maimaiga* resting spores became infected one year after an epizootic but not when resting spores were two years old (Shimazu et al., 1987).

Findings from the present study provide new information about the distribution of the reservoir of *E. maimaiga* in forest soils. These data will help to begin development of sampling plans by identifying the specific areas where the highest densities of resting spores occur. However, further research will be necessary to determine the numbers of trees from which soil must be sampled to quantify *E. maimaiga* resting spore loads over larger areas.

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