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Journal of Invertebrate Pathology 79 (2002) 37–43

Journal of INVERTEBRATE PATHOLOGY

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Deposition and germination of conidia of the entomopathogen *Entomophaga maimaiga* infecting larvae of gypsy moth, *Lymantria dispar*

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Received 24 September 2001; accepted 20 February 2002

Abstract

Germination of conidia of *Entomophaga maimaiga*, an important fungal pathogen of gypsy moth, *Lymantria dispar*, was investigated on water agar and larval cuticle at varying densities. Percent germination was positively associated with conidial density on water agar but not on larval cuticle. When conidia were showered onto water agar, the rate of germination was much slower than on the cuticle of *L. dispar* larvae. From the same conidial showers, the resulting conidial densities on water agar were much higher than those on larval cuticle in part because many conidia adhered to setae and did not reach the cuticle. A second factor influencing conidial densities on larval cuticle was the location conidia occurred on larvae. Few conidia were found on the flexible intersegmental membranes in comparison with the areas of more rigid cuticle, presumably because conidia were physically dislodged from intersegmental membranes when larvae moved. Conidia were also exposed to heightened CO_2 to evaluate whether this might influence germination. When conidia on water agar were exposed to heightened CO_2 levels, germinating conidia primarily formed germ tubes while most conidia exposed to ambient CO_2 rapidly formed secondary conidia.

Keywords: *Entomophaga maimaiga*; *Lymantria dispar*; Entomopathogenic fungi; Entomophthorales; Germination; Autostimulation; Infection processes; Biological control

1. Introduction

Self-inhibitory and self-stimulatory effects of spore crowding may be important in mediating infection processes (Cooke and Whipps, 1993); however, across the classes of fungi there seems to be no rule about how spore crowding affects germination. Spore density has been shown to increase, decrease, and have no effect on conidial germination. For the Zygomycetes *Mucor plumbeus* and *Rhizopus stolonifer* (Robinson et al., 1968), as well as for *Geotrichum candidum*, an anamorph of a Saccharomycete (Robinson, 1973; Robinson and Thompson, 1982), conidial germination and germ tube growth have been positively correlated with spore density. Conversely, for the hyphomycete *Aspergillus nidulans*, a negative correlation was found between conidial density and germination (Trinci and Whittaker, 1968). Crowding the uredospores of the rust *Uromyces phaseoli* decreased total germination but increased average germ tube growth (Yarwood, 1956). For the entomophthoraleans *Erynia conica* and *Erynia curvispora*, density did not influence conidial germination or the type of secondary conidia produced (Nadeau et al., 1996b).

It has been hypothesized that density dependent stimulation and self-stimulation of conidial germination due to crowding could be caused by the CO_2 produced when spores respire (Cooke and Whipps, 1993). In agreement, germination of *A. nidulans* conidia is enhanced by the presence of CO_2 (Trinci and Whittaker, 1968).

The entomophthoralean *Entomophaga maimaiga* is a virulent Asian pathogen of gypsy moth, *Lymantria dispar*, larvae (Hajek, 1999). Although intentionally introduced to North America in 1910 and 1911, this fungus

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0022-2011/02/$ - see front matter
PII: S0022-2011(02)00010-1

has only been found abundantly in North America since 1989. Whether *E. maimaiga* persisted from initial introductions or was accidentally introduced at a later date remains in question (Hajek et al., 1995b). Due to interest in preserving the invertebrate fauna of North American forests as well as in manipulating *E. maimaiga* for biological control of *L. dispar*, the host specificity of this fungus has been evaluated in the laboratory and field (Hajek et al., 1995a, 1996, 2000). To continue these studies toward an understanding of determinants of host specificity, we investigated factors potentially influencing the activity of *E. maimaiga* conidia on host cuticle. We tested whether conidial density affected germination, both on water agar and on larval cuticle. Although we assume that conidial densities encountered in the field are not often high, high densities can readily be generated under laboratory conditions so it is important to know whether conidial density influences germination behavior. In addition, we evaluated the distribution and activity of conidia on host cuticle. Due to evidence that CO_2 levels influence germination of other fungi, we investigated whether heightened CO_2 could influence germination behavior of *E. maimaiga*.

2. Materials and methods

2.1. Experimental organisms

The isolate of *E. maimaiga* used for density studies was ARSEF 5569, collected in 1996 from infected *L. dispar* larvae in Virginia, USA. For CO_2 studies, ARSEF 6162, isolated from soil collected in Japan in 1998, was used because the original Virginian isolate no longer would reliably produce conidia. These isolates are maintained in the USDA, ARSEF (Agricultural Research Service Collection of Entomopathogenic Fungal Cultures) in Ithaca, NY. Protoplasts of *E. maimaiga* were grown in 95% Grace's insect tissue culture media (GIBCO-BRL, Gaithersburg, MD), supplemented with 5% fetal bovine serum (GIBCO-BRL) at 20 °C in the dark.

L. dispar larvae were obtained as neonates from the USDA, APHIS, Otis Methods Development Center, MA and were reared on high wheat germ diet (Bell et al., 1981) at 23 °C, 14:10 (L:D) in groups of 10 in 236 ml plastic cups.

2.2. Protocol for showering conidia

Although it is possible for *E. maimaiga* grown in vitro to produce conidia upon removal from media, conidial discharge generally occurs slowly over a long period of time. In contrast, conidial discharge from cadavers of *E. maimaiga*-killed *L. dispar* larvae can be dense for shorter time periods. For these experiments abundant conidial showers were needed for prescribed periods of time, and therefore cadavers of recently dead *E. maimaiga*-killed *L. dispar* larvae were used to produce conidia.

To obtain sporulating *L. dispar* cadavers, fourth instar larvae were injected with 10 μl of a 1.0×10^5 protoplast/ml suspension of two-day old *E. maimaiga* protoplasts in Grace's insect tissue culture media and then maintained at 20 °C, 14:10 (L:D) until death. Insects were monitored daily between days 4 and 6 post-injection and resulting cadavers were transferred to 100% RH at 20 °C to initiate fungal outgrowth and conidial discharge.

To shower conidia for experiments, cadavers were placed over either water agar or *L. dispar* larvae at 100% RH and 20 °C in darkness. Ten to thirty *L. dispar* cadavers with abundant outgrowth of conidiophores were placed, dorsal side down, on cheesecloth to support them. The cheesecloth was then placed over 15×15 cm hardware cloth platforms (1.2-cm mesh) for conidial showers.

2.3. Conidial density vs. germination on water agar

Sporulating cadavers were placed 2 cm above 60×15 mm polystyrene petri dishes containing 1% water agar (Difco Labs, Detroit, MI) within a larger container at 100% RH. One percent water agar was used because at this concentration germinating conidia of this isolate (ARSEF 5569) primarily produced germ tubes and not secondary conidia. To achieve a diversity of conidial densities, conidia were showered through well-separated windows, and 0, 2, 4, or 6 layers of plastic screens (mesh size $= 0.96 \times 0.96$ mm) were present between sporulating cadavers and the water agar in each dish. Every 30 min, petri dishes were rotated beneath the cadavers to ensure an even distribution of conidia from the different showering cadavers. Cadavers showered conidia over a 3-h period. After showering, water agar plates were inverted to prevent contamination by secondary conidia.

Conidia deposited onto water agar were observed at 128× using an Olympus SZH dissecting microscope. To quantify conidial density, the numbers of conidia within an ocular grid (750×750 μm) were counted for 10 grids for each area of a petri dish that had been covered by a different number of screens. Almost all conidia produced only one germ tube and a conidium was considered germinating if the length of the germ tube was greater than a quarter of the width of the conidium. To assess production of germ tubes, a minimum of 100 conidia were quantified for each density area. Counts were made at 4 and 24 h post-inoculation for each of the four densities, with three replicates on each of four dates.

2.4. Conidial density vs. germination on L. dispar larvae

One percent water agar was poured to within 1–2 mm of the top of two 60×15 mm polystyrene petri dishes

and 6–10 third instar *L. dispar* larvae were placed on top of the water agar in each dish. To prevent larvae from escaping, plastic screening was placed over each dish. Four additional plastic screens were placed 1 cm above one of the petri dishes to achieve a lower density conidial shower on the larvae in that dish. By the time these studies were conducted, we had devised a motorized method to ensure that conidia from individual cadavers were equally showered onto all surfaces. Petri dishes containing larvae were placed on a 150-mm diameter turntable that rotated at 2 rpm 5 cm below the platform supporting the sporulating cadavers. This assembly was maintained at 20 °C, 100% RH in darkness for 3 h while cadavers showered conidia. As controls, conidia were showered onto 60 × 15 mm petri dishes containing water agar, as for in vitro assays.

Because the conidia showered onto larvae germinated much more quickly than those on water agar, intervals after which germination was monitored were shortened. At 2 and 4 h after the 3-h showering period, larvae were immobilized until scoring by placing them in a covered glass dish containing a chloroform-soaked tissue at 4 °C. Due to this method of immobilization, the same larvae could not be used for both 2- and 4-h counts. Only conidia on the dorsal surfaces of larvae were observed. Conidial density, percent germination, and germ tube length were quantified for a minimum of 30 fields of view at 128× for 3–10 insects for each replicate of the experiment. Larvae with less than 20 conidia total in the 30 fields of view were excluded from the experiment.

We noted that conidial density varied drastically among different zones of the larval cuticle and conidial densities were therefore quantified separately for each zone. The dorsal cuticle of *L. dispar* was divided into three distinct zones (Fig. 1): (1) verrucae, the sclerotized

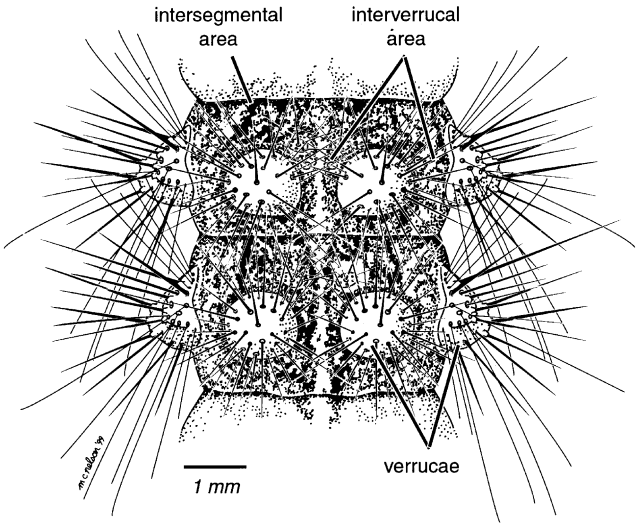

Fig. 1. Schematic of the dorsal view of segments 3–6 of a late instar *Lymantria dispar* larva.

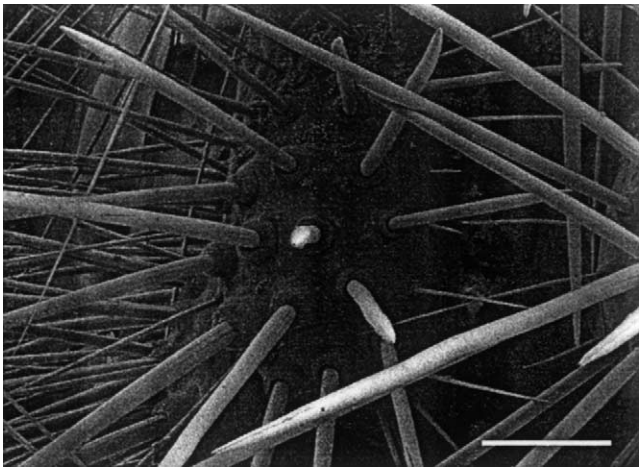

Fig. 2. Scanning electron micrograph of an *L. dispar* verruca. Scale bar = 500 μm.

protuberances bearing large secondary setae (Fig. 2), (2) cuticle surrounding verrucae (interverrucal area), and (3) intersegmental membranes. Therefore, conidia were counted in 10 fields of view for each of these zones in each of the three replicates of this experiment.

2.5. CO_2 studies

To determine the effect of CO_2 on conidial germination, *E. maimaiga* conidia were showered onto 1.5% water agar in 35 × 10 mm petri dishes using the rotating platform. Conidial showers usually lasted ca. 30 min to obtain a desired density of >15 conidia/mm^2. Petri dishes were then lidded, inverted, and four petri dishes were placed into each of two air-tight, light-tight vessels at 100% RH. Using a vacuum pump, the pressure in the vessels was reduced to about 90% atmospheric pressure (ATM). The 10% was then immediately replaced with either air for the control or pure CO_2 for the treatment. After being returned to ATM, the vessels were placed into a 15 °C incubator where they were left for 48 h. After this exposure period, vessels were opened and conidia were immediately evaluated. During previous studies of density and location, production of secondary conidia had not been abundant, but during the CO_2 studies, using a different isolate (ARSEF 6162) on water agar that was slightly harder than that used during density studies (1.5% vs. 1.0%), this type of germination predominated in controls. To quantify responses, conidia were classified as remaining unchanged, forming a germ tube, or forming a secondary conidium. Counts were made at 64× on a dissecting microscope using an ocular grid (1.6 × 1.6 mm). Two to three grids were counted for each of the four petri dishes, yielding 10 grids counted for the control and the CO_2 exposure for each of the three replicates of this experiment.

2.6. Data analysis

For the effect of conidial density on germination on water agar and on larval cuticle, data for different time periods could be merged and were then analyzed using logistic regression. Data from the four replicates were dummy-coded for this analysis. Analysis of variance was used to compare germ tube length by location on the cuticle as well as differences in the types of germination between CO_2-exposed and control conidia.

3. Results

3.1. Conidial germination vs. density on water agar

Production of germ tubes by conidia on water agar was positively associated with conidial density (Fig. 3). A model adding density squared and controlling for the dates of experimental replication gave an improved regression (conidial density odds ratio = 1.138; square of conidial density odds ratio = 0.999; both $p < 0.001$) (area under the ROC curve (c) = 0.782). Although dates were highly significant and thus differed ($p < 0.001$), the same trend of increased production of germ tubes at higher density was seen for all dates. Percent conidia producing germ tubes on water agar also differed by time, being greater at 24 h than at 4 h (odds ratio = 0.672; $p < 0.001$), demonstrating that germ tube production was incomplete at 4 h. For example, at lower densities of <10 conidia/mm^2, at 4 h, $11 \pm 4\%$ of conidia had germinated while by 24 h, $21 \pm 5\%$ of conidia had germinated.

During this study, conidial densities on water agar varied from 3.2 ± 0.5 to 63.3 ± 6.7 conidia/mm^2 (mean $\pm$ SE). Because conidial showers distributed conidia fairly randomly, at lower overall conidial densities many conidia were distant from each other. In such cases, regardless of the overall density we observed that conidia near their neighbors produced germ tubes more frequently than conidia that were far apart.

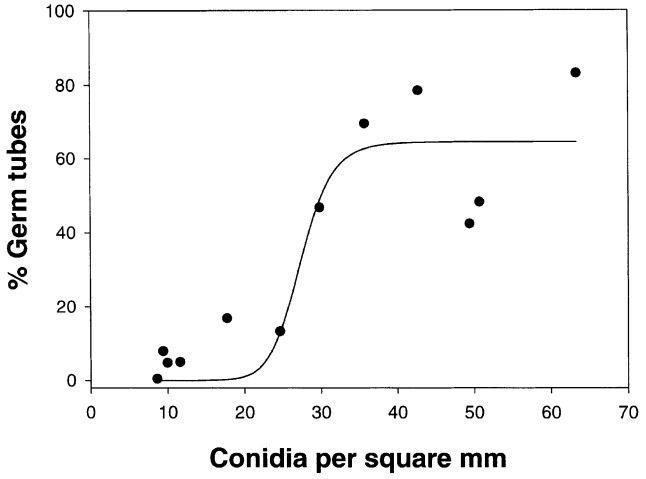

Fig. 3. Relationship between conidial density and percent production of germ tubes for *E. maimaiga* conidia showered onto water agar. Due to variability among replicates, this graph displays 4-h data from only one replicate (non-linear fitted curve; $r^2 = 0.832$).

3.2. Conidial germination vs. density on L. dispar larvae

Contrary to in vitro findings, conidial germination on *L. dispar* larvae was not associated with density (odds ratio = 1.802; $p > 0.05$) or density squared (odds ratio = 1.002; $p > 0.05$) ($c = 0.650$). Data from only one date differed ($p = 0.0137$), but this was due to the lack of low densities for this replicate. Also, in contrast to the in vitro study, conidia on larval cuticle germinated much more quickly. By 4 h, only $19 \pm 18\%$ of conidia on control water agar plates had germinated although $79 \pm 3\%$ of conidia on insect cuticle had germinated.

Although conidia were deposited on cuticle and water agar during the same showers using the rotating platform, densities of conidia reaching the larval cuticle were much lower than densities on water agar (conidia/mm^2 on insect cuticle = (0.15674 × conidia/mm^2 on water agar) + 0.20719; $F = 69.80$; $r^2 = 0.9332$; $p = 0.0004$). While densities on control water agar plates ranged from 1.2 to 21.2 conidia/mm^2, conidial densities on larval cuticle were much lower, ranging from 0.3 to 4.0 conidia/mm^2. As is typical of members of the Lymantriidae, the Lepidopteran family to which *L. dispar* belongs, *L. dispar* larvae have abundant setae. We observed that many conidia showered onto larvae adhered to setae (Fig. 4). Although we were unable to count conidia on setae, the common occurrence of conidia on setae certainly drastically decreases conidial densities reaching the cuticle. Conidial density on larval cuticle also varied by cuticular zone. Conidial densities were low on both intersegmental membranes and verrucae but conidia were more than twice as abundant in the areas surrounding verrucae ($F = 19.76$; $p = 0.0001$) (Fig. 5), although equivalent numbers of conidia must have fallen over all cuticular zones. The verrucae have many setae and it seems plausible that many of the conidia falling above the verrucae attached to the setae. Although intersegmental membranes are not well protected by setae, these areas constantly flex and fold as larvae walk. Larvae would have been walking in dishes for 2 or 4 h after the 3-h showering period. We hypothesize that conidia were rarely found on intersegmental membranes because they were usually dislodged as larvae moved (Fig. 6A,B). Using scanning electron microscopy, in intersegmental membranes at times areas were seen that we hypothesized were either mucous remaining from dislodged conidia or remnants from crushed conidia.

Analyzing percent germination by cuticular area revealed no differences by location (χ^2 tests; all $p > 0.05$).

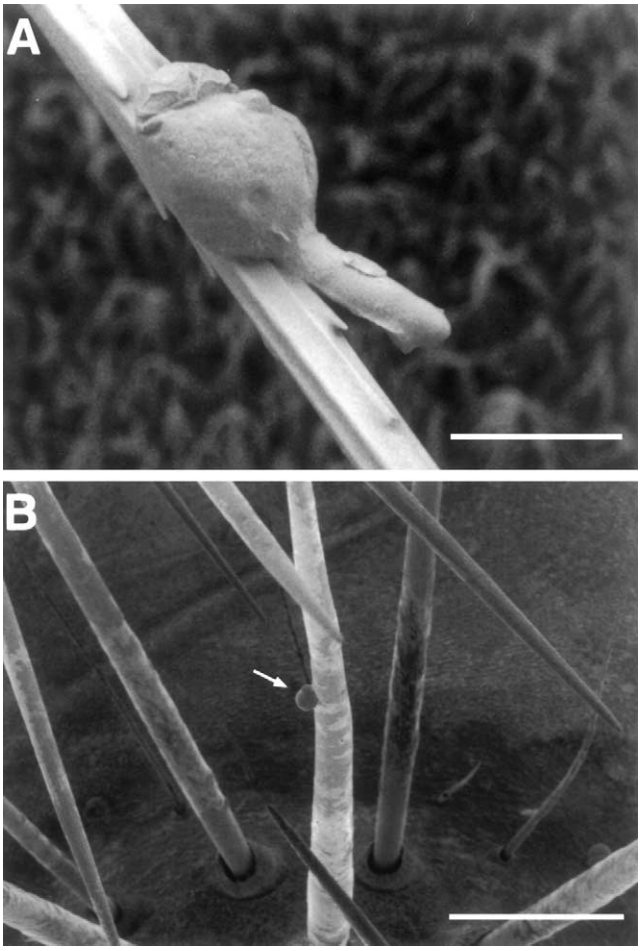

Fig. 4. Conidia showered onto *L. dispar* that have adhered to setae. (A) Scale bar = 20 μm. (B) Scale bar = 150 μm.

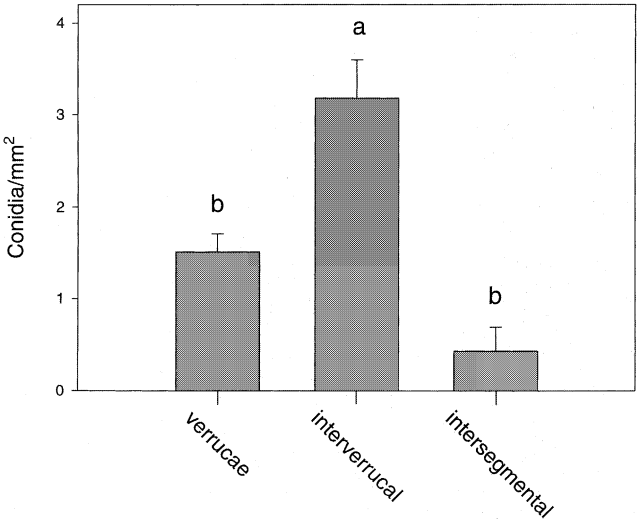

Fig. 5. Densities (mean ± SE) of *E. maimaiga* conidia on *L. dispar* cuticle by differing cuticular regions. Differing letters above bars denote significant differences based on post hoc Sidak inequality tests ($\alpha = 0.05$).

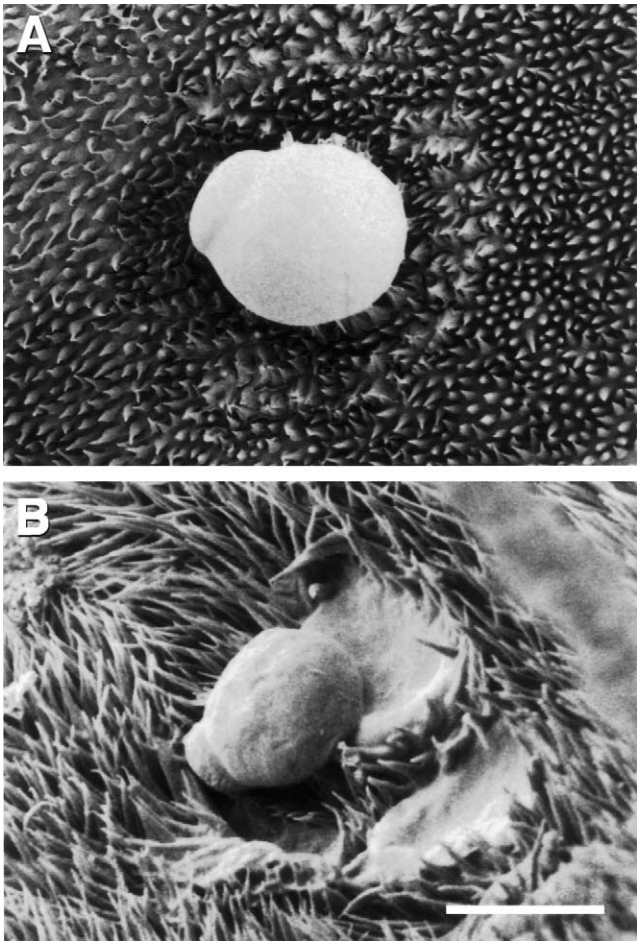

Fig. 6. *E. maimaiga* conidia on the waxy cuticle of larval *L. dispar*. (A) Ungerminated conidium. (B) Dislodged conidium of *E. maimaiga* on an intersegmental membrane of *L. dispar*. The mucous covering the conidium has been smeared as the conidium was dislodged. Scale bar = 20 μm.

Germ tube length was evaluated by location and time and differences were found by time, with longer germ tubes at 4 h (43 ± 6 μm) than at 2 h (24 ± 2 μm) ($F = 6.03$; $p = 0.0163$), but not by location ($F = 1.49$; $p = 0.2324$).

3.3. Effects of CO_2 on conidial germination

There was a significant difference by treatment in the type of growth during germination for conidia exposed to elevated CO_2 vs. air ($F = 3297.55$; $p < 0.0001$) (Fig. 7). By 48 h, most conidia exposed to elevated CO_2 made germ tubes ($97 \pm 2\%$) while virtually none ($0.2 \pm 0.2\%$) exposed to air made germ tubes. In contrast, $100 \pm 0\%$ of conidia exposed to air produced secondary conidia while only $2 \pm 1\%$ exposed to CO_2 made secondary conidia. Production of secondary conidia was more rapid than the formation of germ tubes. By 24 h, $92 \pm 4\%$ of control conidia had formed secondary conidia while only $7 \pm 7\%$ of CO_2-exposed conidia had formed germ

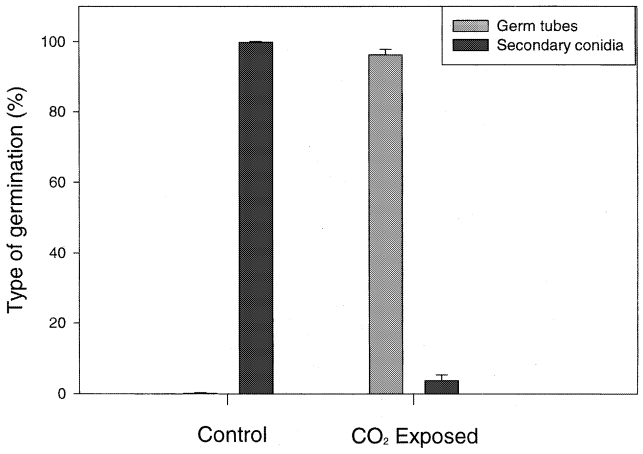

Fig. 7. Percent type of germination by *E. maimaiga* conidia (mean ± SE) on water agar exposed to elevated CO_2 compared with conidia exposed to ambient conditions.

tubes, although by 48 h, 64 ± 20% of CO_2-exposed conidia had formed germ tubes.

4. Discussion

Germination of *E. maimaiga* conidia was positively associated with conidial density when conidia were on water agar, although this same relationship did not occur on host cuticle. In contrast, conidial density had no effect on germination of primary conidia for the entomophthoralean species, *E. conica* and *E. curvispora*, when showered onto water agar although the densities of spores that were tested were not mentioned (Nadeau et al., 1996b). Cooke and Whipps (1993) suggested that the association of increased germination of fungal spores with increasing spore density was possibly an artifact of in vitro studies. Based on our findings, we agree that studies conducted in vitro are unrealistic; the high densities we could create on water agar were never seen on the cuticle of *L. dispar*, in part because these larvae bear abundant setae that prevented conidia from reaching the larval cuticle.

We found that elevated CO_2 strongly influenced the type of growth when conidia germinated as well as the speed of germination. These results are consistent with our findings of increased germ tube production when conidia are more dense in vitro; in these conditions, CO_2 in the vicinity of conidia would most probably be higher due to the greater density of respiring spores. Evidence exists from other fungi implicating volatile metabolites such as O_2 or CO_2 as stimulators or inhibitors of spore germination and germ tube growth (Robinson and Thompson, 1982; Trinci and Whittaker, 1968). We did not try to replicate the actual concentration of CO_2 that occurs on the larval cuticle and the extent to which CO_2 influences spores on the complex larval cuticle is not known. If the germination behavior of *E. maimaiga* conidia is indeed influenced by volatiles, the surface of *L. dispar* larvae may help to play a role in explaining the rapid onset of spore germination on living larvae compared with water agar. Abundance of setae has been shown to create an insulated area around *L. dispar* larvae (Casey and Hegel, 1981); CO_2 and other volatiles could therefore potentially be concentrated to some extent near the cuticle surface.

During this study conidia on host cuticle produced germ tubes and only very rarely produced secondary spores. With heightened exposure to CO_2, conidia on water agar also made germ tubes instead of secondary conidia. However, production of germ tubes from conidia exposed to heightened CO_2 was much slower than production of secondary conidia on water agar. The production of secondary conidia on water agar with ambient levels of CO_2, presumably when other potential cues present on cuticle were absent, suggests a rapid escape response by this fungus when on an inappropriate surface, providing another chance to disperse and find a host.

Retention of conidia on larval cuticle was strongly influenced by the location on the insect where spores landed. We saw evidence of conidia in intersegmental membranes being dislodged or even crushed and conidia landing in the flat, non-bending interverrucal zones were at higher densities. Cuticular topography has previously been shown to aid spores in successful penetration of insect cuticle (Wraight et al., 1990). Here, we have found that cuticular topography also is associated with the distribution of spores. It seems that the abundant long setae of *L. dispar* larvae protected larvae to some extent from conidia landing on the cuticle, yet some conidia were still able to reach the cuticle. Although relatively few conidia breached the setal barrier, the effectiveness of setae in preventing infections is questionable; studies have shown that for other species of Entomophthorales, only a few conidia are needed to initiate a successful infection (Wraight et al., 1990).

Conidia on the larval cuticle produced germ tubes very rapidly when compared with conidia on water agar, suggesting that stimulants are present. Rapid germination on hosts has also been documented for *E. conica* on black flies (Nadeau et al., 1996a) and *Zoophthora radicans* (=*Erynia radicans*) on leafhoppers (Wraight et al., 1990). The nature of the stimulants in the *L. dispar*/*E. maimaiga* system is presently being investigated further. For other species of Entomophthorales, cuticular lipids are known to influence conidial germination (Boucias and Latgé, 1988; Kerwin, 1984; Latgé et al., 1987; Nadeau et al., 1996a).

Importantly, we have shown that conidial density can have a dramatic effect on the percent of conidia producing germ tubes on water agar. While the occurrence of these density effects in nature is not known, they re-

main important in the laboratory, where significant effects could be introduced to experiments conducted in vitro if conidial density is not controlled.

Acknowledgments

We thank M. Bertoia, N. Bertoia, B. Knoblauch, J. MacNeil, and M. Wheeler for rearing *L. dispar* larvae during this study, D. Ewing for assisting with production of conidia, M.C. Nelson for her expertise drawing Fig. 1 and I. Delalibera for insights during this study. This study was supported in part by USDA, NRICGP #96-35312-3883.

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