

ENTOMOPATHOGENIC FUNGI CARRIED BY THE CAVE ORB WEAVER SPIDER, *META OVALIS* (ARANEAE, TETRAGNATHIDAE), WITH IMPLICATIONS FOR MYCOFLORA TRANSFER TO CAVE CRICKETS

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Abstract: We report the presence of the entomopathogenic fungi, *Beauveria* spp. and *Paecilomyces* spp., associated with female adults of the cave orb weaver spider, *Meta ovalis*, from Laurel Cave (Carter Cave State Resort Park, Carter Co., Kentucky). There was also an abundance of saprophytic *Aspergillus* spp., *Mucor* spp., *Penicillium* spp., *Rhizopus* spp., and to a lesser extent, *Absidia* spp., *Cladosporium* spp., *Mycelia sterilia*, and *Trichoderma* spp. These are mostly saprobes that reflect the mycoflora that are typical of the cave environment. Incubation at 25 °C resulted in increased growth of all fungi compared to growth at 12 °C (cave conditions) on each of four different kinds of culture media, indicating that the cave environment is suppressive for the growth of these fungi. Topically-applied inocula of *Beauveria* sp. and *Paecilomyces* sp. (spider isolates) were not pathogenic to *M. ovalis*, but these fungi were pathogenic to the cave cricket, *Hadenoeus cumberlandicus*. One possibility is that the *Beauveria* spp. and *Paecilomyces* spp. carried by *M. ovalis* could negatively impact the survival of cave crickets that co-occur with these spiders, thus possibly altering the ecological dynamics within the caves.

INTRODUCTION

A mycological survey of the cave orb weaver spider, *Meta ovalis*, was done to provide information related to factors that may influence spider population dynamics. We determined which fungi may be potential spider pathogens (internal mycoflora) and which may be spread to other cavernicolous invertebrates (external mycoflora), especially cave crickets. Our study was done in Laurel Cave (38° 22' 30.8" N, 83° 06' 55.4" W), in the cold, dry upper level (Pfeffer et al., 1981). This is a small cave (total length 1019 m) developed in Mississippian limestone and located in Carter Cave State Resort Park, Carter Co., Kentucky where these spiders have been found to be particularly abundant. *M. ovalis* occurs most commonly in the entrance and twilight zone of Laurel Cave and occasionally in the deeper parts of the cave. Whether migration occurs between cave and epigeal environments is not known, but they can be found outside on occasion. We do not usually observe individuals of *M. ovalis* especially close to one another; they appear to be inactive most of the time, hanging attached to some part of the web, seemingly more on the periphery, but whether this is the preferred placement on the web is not known. Little, if any, information exists on their reproductive cycle or life history. They are not classic cave-adapted organisms that are K-strategists that produce a very small number of offspring (Hobbs, 1992); rather, they produce a sizeable egg case (exact number of eggs is not known) with numerous offspring, thus being more characteristic of r-

strategists. Exact data on their longevity are lacking, but we speculate that they have a fairly long life (Lavoie et al., 2007).

In Laurel Cave, individuals of *M. ovalis* are virtually never found on the cave floor or low on cave walls and appear to prefer ceilings and upper walls in areas that are recessed (termed kettles or bells) and out of the desiccating impact of air currents. Often, these spiders are in close proximity to cave crickets (both *Ceuthophilus* spp. and *Hadenoeus* spp.), sometimes as close as several centimeters, with no apparent impact on crickets (Yoder et al., 2009) even though they are regarded as cricket predators. Millipedes, other spiders, and carabid beetles are other possible prey (Lavoie et al., 2007). *Hadenoeus* spp., in particular *H. cumberlandicus* in Laurel Cave, are notoriously found in caves and have importance as a keystone species by supplying food (guano) to an array of millipedes, flies, and beetles that reside under the cricket roost (Lavoie et al., 2007). Dense aggregations, consisting of up to hundreds of individuals, are the hallmark behavior of *Hadenoeus* spp. (Studier et al., 1986; Yoder et al., 2002). In Laurel Cave, *M. ovalis* is reliably found close by these cricket aggregations (Yoder et al. 2009), which makes *M. ovalis* relevant for influencing the cave ecosystem by impacting the cave crickets.

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Table 1. External mycoflora of cave orb weaver spider, *Meta ovalis*, from Laurel Cave (Carter County, Kentucky, USA). NA, nutrient agar; BA, blood agar; MMN, modified Melin-Norkrans agar; PDA, potato dextrose agar.

Fungi	No. external isolates from <i>Meta ovalis</i> (n = 40 spiders)					
	NA	BA	MMN	PDA	Total	%
<i>Absidia</i> spp.	1	0	2	1	4	8.3
<i>Aspergillus</i> spp.	1	1	5	4	11	22.9
<i>Beauveria</i> spp.	0	0	0	2	2 ^a	4.2
<i>Cladosporium</i> spp.	1	0	1	0	2	4.2
<i>Mucor</i> spp.	1	2	2	2	7	14.6
<i>Mycelia sterilia</i>	0	0	2	2	4	8.3
<i>Paecilomyces</i> spp.	0	1	1	0	2 ^a	4.2
<i>Penicillium</i> spp.	2	1	2	2	7 ^a	14.6
<i>Rhizopus</i> spp.	2	1	3	2	8	16.7
<i>Trichoderma</i> spp.	0	0	1	0	1	2.1
Total	8	6	19	15	48	

^a Fungus that was also isolated internally.

METHODS

A total of 40 spiders were used; ten each for four different embedding media, with five being used for external isolations and five being used for internal isolations. The test group of 40 spiders represents several collecting trips, 2004–2007, to prevent depleting the population, but they were collected during the same time of year, August–November. The test groups were divided, one for growth at 12 °C and the other at 25 °C.

Standard aseptic technique was followed for spider collection, and specimens were plated less than 12 h after collection. Only female adults spiders were used (identified according to Ubick et al., 2005). Embedding media were blood agar-lyophilized bovine blood (BA), modified Melin-Norkrans agar (MMN), nutrient agar (NA) and potato dextrose agar (PDA) to maximize recovery of fungi that might be fastidious. Incubation conditions were 12 ± 1 °C (to mimic Laurel Cave; Hill, 2003) in total darkness.

Preparation of samples for external fungus recovery involved placing a spider into a Petri dish, covering with molten embedding media (one dead spider per dish), incubation, daily examination (100×) for hyphae (typically 5–10 days), excising a single hyphal tip by a scalpel (100×, tracing hypha to spider's body surface), placing the agar block containing tip on a fresh plate of solidified media, and daily examination until culture characteristics appeared for identification (criteria of Barnett and Hunter, 1998). Isolates that failed to produce identifiable structures were designated as *Mycelia sterilia*; sterility is common for cave fungi due to unchanging environmental conditions and darkness (Chapman, 1993).

The procedure for internal fungus recovery was similar, except the spider was washed (twice for 1 minute each) in sterile deionized (DI) water, then mild bleach solution (DI water-absolute ethanol-5.25% NaOCl; 18-1-1, v/v/v) followed by two DI water rinses, and the body was quartered

(one body portion/plate) prior to embedding (Currah et al., 1997; Zettler, 1997; Yoder et al., 2003).

The radial growth rate of each fungus was determined using the trisecting line method as described by Currah et al. (1997). Three lines were drawn on the bottom of a Petri dish radiating from the center of the dish 40° from each other. The dish was filled with agar and allowed to solidify. A 1 cm³ block of mycelium from an established culture was placed on the agar surface in the center over top of the point of intersection of the three lines and incubated (12 °C and 25 °C, darkness). Each day for the next five days, measurements were taken along each of the three lines as the mycelium spread over the agar surface. To calculate the radial growth rate (K_r), we used the equation $K_r = (R_1 - R_0)/(t_1 - t_0)$, where R_0 and R_1 are colony radii at initial (t_0) and elapsed (t_1) times between beginning of linear, t_0 , and stationary, t_1 , growth phases as described by Baldrian and Gabriel (2002). The radial growth rate was expressed as millimeters per hour. For each fungus, data are mean ± standard error (SE) of 45 measurements, 15 per plate, replicated three times each using mycelia selected randomly from ten separate pure cultures of a particular fungus. An analysis of variance (ANOVA) was used to compare data (SPSS 14.0 for Windows, Microsoft Excel and Minitab; Chicago, IL) as described by Sokal and Rohlf (1995).

Inocula were prepared from spider isolates *Beauveria* sp. and *Paecilomyces* sp. (Table 1), genera that are classified as entomopathogens (Barnett and Hunter, 1998), and tested to determine whether these fungi are infective to spiders and cave crickets, *H. cumberlandicus*. An aqueous inoculum was made by shaking a 1 cm³ block of mycelium from established cultures in 5 ml phosphate-buffered saline (PBS, pH 7.5) overnight. Conidia concentration was adjusted to 6.8×10^8 conidia ml⁻¹ (20 µl application) per individual (modified from Kirkland et al., 2004) using PBS based on 10 separate counts with a hemocytometer (AO Spencer Bright Line, St. Louis, MO)

and 0.1% trypan blue exclusion; PBS served as a control. Each fungus formulation (and PBS control) was applied topically to spiders ($n = 20$, 4 replicates of 5, with each replicate representing a different collecting trip) and crickets ($n = 30$, 5 replicates of 6, with each replicate representing a different collecting trip). Specimens were housed in individual clear 8000 cc plexiglass chambers at 12 ± 0.5 °C, $98 \pm 2.0\%$ relative humidity (RH) and darkness. The chamber was inverted so that crickets could be in an upside down position like they are in the cave. The criteria used to determine a dead or dying cricket or spider were lack of movement, failure to respond to mechanical stimuli, and inability to right and crawl five body lengths. Observations were made daily in red light. Data were compared using ANOVA.

RESULTS AND DISCUSSION

A listing of external and internal fungi associated with *M. ovalis* is presented in Table 1. The percentages represent the diversity and amount of fungi that may be found on a single individual spider at any one time. Fungi from ten genera were recovered from the spider's body surface, with *Aspergillus* spp., *Mucor* spp., *Penicillium* spp., and *Rhizopus* spp. as major isolates. All of the fungi in the spider's external mycoflora are common, naturally-occurring fungi present in soil, leaf litter, and organic debris, where they function as agents of decay (Cubbon, 1976; Rutherford and Huang, 1994; Reeves et al., 2000). Incubation at 12 °C, reminiscent of mean temperature in the interior of Laurel Cave, had a suppressive effect on growth/spread of the mycelium and delayed sporulation (production of conidia) of all of these fungi; that is, it took about twice as long compared to incubation at 25 °C for culture characteristics to appear. Radial growth rate (mean \pm SE < 0.034) at 25 °C and at 12 °C dropped from 0.088 mm h⁻¹ to 0.031 mm h⁻¹ for *Aspergillus* sp.; 0.308 mm h⁻¹ to 0.114 mm h⁻¹ for *Mucor* sp.; 0.127 mm h⁻¹ to 0.055 mm h⁻¹ for *Penicillium* sp. and 0.367 mm h⁻¹ to 0.208 mm h⁻¹ for *Rhizopus* sp. as the predominate isolates on the spider's surface ($p < 0.05$). Less frequently recovered fungi from the spider's surface showed similar rate reductions in response to low temperature: 0.224 mm h⁻¹ (25 °C) vs. 0.125 mm h⁻¹ (12 °C) for *Absidia* sp., 0.197 mm h⁻¹ (25 °C) vs. 0.073 mm h⁻¹ (12 °C) for *Beauveria* sp.; 0.141 mm h⁻¹ (25 °C) vs. 0.0884 mm h⁻¹ (12 °C) for *Cladosporium* sp.; 0.239 mm h⁻¹ (25 °C) vs. 0.118 mm h⁻¹ (12 °C) for *Paecilomyces* sp.; and 0.313 mm h⁻¹ (25 °C) vs. 0.146 mm h⁻¹ (12 °C) for *Trichoderma* sp. The ubiquitous distribution of the fungi identified from the spiders is reflected by their ability to grow on a variety of different culture media (Table 1), showing no particular nutritional or pH requirement consistent with their ability to utilize a variety of substrates for growth and proliferation (Jennings and Lysek, 1999).

Noteworthy among the spider body surface isolates were *Beauveria* spp. and *Paecilomyces* spp. that are

classified ecologically as facultative parasites, which under certain conditions can switch from saprobe to parasite (but classified as obligate parasites according to Samsinakova et al., 1974), and act as insect pathogens. *Beauveria* spp., in particular, is often used in biological control (Hajek and Butler, 2000; Strasser et al., 2000). *Trichoderma* spp. is commonly mycoparasitic (Jennings and Lysek, 1999). Because Laurel Cave is stable at low temperature (12 ± 1.1 °C) and high relative humidity (98.6–100% RH) (Hill, 2003), these conditions favor a small number of prolific fungal taxa, especially anamorphs, such as those isolated (Table 1), namely *Aspergillus* spp. and *Penicillium* spp. that consistently show high abundance in caves (Cubbon, 1976; Rutherford and Huang, 1994; Reeves et al., 2000). We observed no appreciable difference in mycoflora from spiders collected from different years (data not shown). Therefore, the fungi isolated from the body surface of *M. ovalis* are typical of a cave setting, all are accelerated conidia-producing genera allowing rapid dispersal, and a heavy, diverse fungus load can apparently be supported by the spider without any noticeable detrimental effects or changes in spider appearance or behavior.

Of special interest was the internal isolation, albeit low, of the entomopathogens *Beauveria* spp. and *Paecilomyces* spp. from within *M. ovalis* body contents, consistent with their parasitic nature (Table 1). Twenty-three percent of spiders (9 out of 40 spiders) were infected with *Beauveria* spp., and 10% of spiders (4 out of 40 spiders) were infected with *Paecilomyces* spp. As such, recovery of these fungi internally from tissues implies penetration of fungal hyphae, exploiting the spider internally and proliferating inside, presumably originating from the body surface where they were also isolated (Table 1). Gaining access to the inside of the spider's body likely occurs through the mouth, anus, genital, or glandular openings or directly through the cuticle by secretion of proteolytic and chitinolytic enzymes that characterize entomopathogenic infections (St. Leger et al., 1998). The other fungus that was isolated internally (1/40 spiders, 3%) was *Penicillium* spp., classified as a saprobe (Jennings and Lysek, 1999), and this fungus was probably present as a secondary invader or a contaminant; *Aspergillus* spp. and *Mucor* spp. also have this ability (Gliński and Buczek, 2003), but neither of these fungi were detected in our study by internal fungus culture. Evidence from internal recovery from *M. ovalis* of known pathogenic fungi suggests that *Beauveria* spp. and *Paecilomyces* spp. may serve as natural regulators of spider populations in Laurel Cave. Another alternative is that the presence of these fungi internally may be contaminants that could have come from the digestive tract that is contiguous with the outside. Both *Beauveria* spp. and *Paecilomyces* spp., although both well-known insect pathogens, have been shown to be pathogenic to spiders on occasion, but results are inconsistent (i.e., that these fungi are pathogenic to spiders are from the observations of Muma (1975) and that they are not pathogenic to

spiders are from the observations of Baltensweiler and Cerutti (1986)).

In contrast, *Beauveria* spp. and *Paecilomyces* spp. have been shown to be lethal to the cave cricket, *Troglophilus neglectus* (Gunde-Cimerman et al., 1998). We attempted to clarify the parasitic nature of *Beauveria* spp. and *Paecilomyces* spp. in our pathogenic testing of these fungi on spiders and cave crickets as it applies to Laurel Cave. With regard to the spider (mean \pm SE), *M. ovalis*, we observed $20 \pm 3.2\%$ mortality for spiders that had received a topical application of *Beauveria* sp.; $15 \pm 4.4\%$ mortality for spiders treated with *Paecilomyces* sp.; $30 \pm 1.3\%$ mortality for spiders treated with PBS; and $20 \pm 2.7\%$ mortality for spiders that received no treatment. No significant differences were noted between control spiders and spiders that had been treated with fungus (Abbott correction; $p > 0.05$) and no fungi (*Paecilomyces* spp. or *Beauveria* spp.) were recovered internally from those spiders that were dead.

Significant differences, however, were noted between controls and fungus formulation applications in the cricket *H. cumberlandicus* (Abbott correction; $p < 0.05$): $77 \pm 5.3\%$ were observed dead after treatment with *Beauveria* sp. and $90 \pm 4.2\%$ were killed with *Paecilomyces* sp., compared to $23 \pm 2.9\%$ mortality for crickets treated with PBS and $30 \pm 6.4\%$ mortality in untreated controls. *Beauveria* spp. and *Paecilomyces* spp. were recovered by internal fungus culture from the dead crickets. Prior to topical treatment with these entomopathogenic fungi, the crickets were in healthy conditions and showed no signs of disease, implying that they were not previously infected. Conceivably, the co-occurrence of *M. ovalis* with cave crickets (*H. cumberlandicus*) puts the crickets at potential elevated risk of becoming infected, and this is likely enhanced behaviorally by formation of cricket aggregations (enabling increased spread of conidia and exposure to a larger number of individuals at a time). Because these crickets are closely related (only parthenogenic population exists in Laurel cave; Hubbell and Norton, 1978), that may make them more prone to infection. Thus, it seems reasonable to suggest that given the close spider-crickets co-occurrence in Laurel Cave, there is a potential for cross-infection that poses a special risk for cave cricket populations (Benoit et al., 2004).

CONCLUSIONS

Adult females of the cave orb weaver spider, *Meta ovalis*, have fungi on their surfaces and have the potential to disperse fungal spores (conidia) throughout the cave environment. Fungi present are typical of those in a cave setting and most are common filamentous soil saprobes (listed in order of relative abundance): *Aspergillus* spp., *Rhizopus* spp., *Penicillium* spp. = *Mucor* spp., *Absidia* spp., *Beauveria* spp. = *Cladosporium* spp. = *Paecilomyces* spp., and *Trichoderma* spp. A topically-applied *Beauveria* sp. and *Paecilomyces* sp. (well known entomopathogenic fungi) isolated from these spiders were infective and killed

adult cave crickets, *Hadenoeus cumberlandicus*, but these fungi were not pathogenic when applied to the spiders. The fact that *M. ovalis* and *H. cumberlandicus* readily co-occur in caves puts *H. cumberlandicus* at an elevated risk for disease by fungal pathogens.

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