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INTERNAL REPORT: ESTUARINE INVERTEBRATE TESTING OF THE ENTOMOPATHOGENIC FUNGUS, *PAECILOMYCES FUMOSOROSEUS* APOPKA STRAIN 97

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Estuarine Invertebrate Testing of the Entomopathogenic Fungus, *Paecilomyces fumosoroseus* Apopka strain 97

by

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Abstract

Paecilomyces fumosoroseus conidiospores were evaluated for toxicity and pathogenicity to *Mysidopsis bahia* and pink shrimp, *Penaeus duorarum*. Duplicate, static, acute 96-h tests were conducted with ≤24-h-old *M. bahia* (N=10) using viable and heat-killed conidiospores. The low mortalities (≤20 %) observed in control and experimental treatments suggested that *P fumosoroseus* conidiospores were neither toxic nor pathogenic to mysids. Post-larval (juvenile) *P. duorarum* (N=15) were fed a diet containing conidiospores and mycelia of *P. fumosoroseus*. The fungus was recovered from feces of shrimp that were fed viable fungus, but after 30 days no mortalities were observed. All shrimp appeared healthy with no visible signs of disease.

Materials and Methods

Cultivation of Fungus and Recovery of Spores

P fumosoroseus (ATCC 20874) Pa 97 10-II was obtained from Dr. L. S. Osborne, University of Florida, Central Florida Research and Education Center, Apopka, FL, and cultured at 25^0 C on glucose-yeast extract-basal salts medium (GYBS; Boucias *et al* 1988). Solid GYBS medium was prepared by the addition of 2.0% agar. For mysid testing purposes, conidiospores were scraped from the surface of 7-day-old sporulating cultures and suspended in filtered (0.22μm) seawater at a salinity of 20‰ by gentle aspiration in a hand-held tissue homogenizer.

For the shrimp feeding experiment, 1 liter wide-mouth Erlenmeyer flasks containing 200 ml of GYBS were inoculated with a loopful of conidiospores and incubated at 25^0 C for 10- 14 day without shaking. Mycelial mats containing conidiospores were harvested by filtration through Whatman #1 filter paper and stored at -20^0 C. For both assays, a hemocytometer was used to estimate conidiospore densities. Viable counts were estimated by diluting spores or mycelial mats in sterile distilled water containing 0.03% Triton X-100®[1] (Union Carbide, Indianapolis, IN, USA), spreading the dilutions onto GYBS agar plates, and counting colonies which appeared after a 5-day incubation.

P fumosoroseus was cultured from shrimp feces by streak plating freshly- collected

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[1]Mention of tradenames or commercial products does not imply endorsement by the U S Environmental Protection Agency

fecal strands on GYBS agar plates containing 0.5 mg/ml nalidixic acid to suppress bacterial growth. Fungal colonies arising from these plates were identified as *P fumosoroseus* by the size, shape, color and arrangement of the spores.

Mysid tests

Mysids, ≤24-h-old, were obtained from laboratory cultures. Seawater for culture and testing was pumped from Santa Rosa Sound near Gulf Breeze, Florida, filtered to 20μm, diluted to a salinity of 20‰ with dechlorinated tap water, aerated and maintained at 25^0 C.

Techniques used for conidiospore exposures were similar to the Toxic Substances Control Act Guidelines: Final Rules (USEPA 1985). Controls included uninoculated seawater and a heat-killed (autoclaved 20 min, 15 lb/in^2) conidiospore treatment. Sterilization of conidiospores was verified by spread plating 0.1 ml of the suspension on GYBS agar plates and scoring for growth after 5 days of incubation. Exposure vessels were 500 ml glass culture dishes filled with 200 ml of seawater containing suspensions of the conidiospores. Five mysids were added to each exposure vessel using two vessels for each control and eacch conidiospore density. Exposure vessels were covered and incubated with aeration at 25^0 C under a photoperiod of 14-h light:10-h dark. Each day mysids were counted, dead animals were removed and the remaining animals were fed *Artemia* (48 h post-hydration, ≥30 *Artemia*/mysid/day). Salinity, dissolved oxygen (D.O.) and pH were measured daily. Temperature was recorded continuously.

Shrimp feeding experiment

Post-larval pink shrimp were obtained from spawned, eye-ablated adult *Penaeus duorarum* according to the methods of Cripe (1996). Four 64-liter glass aquaria were used for *P. fumosoroseus* feeding tests, two for the heat-killed control and two for the viable fungus. Each aquarium contained 15 shrimp. To prevent cannibalism, each shrimp was confined to a small cage constructed from a 10 cm diameter glass Petri dish with a nylon screen collar (1 mm I.D. mesh) attached with silicone sealant. Flow-through seawater (as described in the previous section) was provided to each aquarium at 1.8 liters/hour. Photoperiod was 12-h light:12-h dark and dissolved oxygen was monitored weekly using a YSI Model 57 oxygen meter. Shrimp were allowed 4 days to acclimate to test aquaria before experimental diet was provided. During the acclimation period each shrimp was fed 5 pellets per day of Shrimp Production 45/10® (Rangen, Inc., Buhl, Idaho). The test was initiated by placing shrimp on experimental and control diets. Diets were prepared by adding viable or heat-killed mycelial mats (0.41 g) and Tetramin® flake fish food (10 g) to 500 ml of 2% agar that had been sterilized in an autoclave (20 min, 15 lb/in^2) and cooled to 39^0 C. This mixture was solidified in sterile plastic Petri plates and stored at 4^0 C. On alternate days each shrimp was fed a cube (0.25 cm^3) of the appropriate test diet. Before feeding, fecal strands and uneaten food were removed from the bottom of each cage. In addition, three cages were rotated from the front to the rear of the aquarium to eliminate position effects during exposure.

Results and Discussion

Mysid tests

Water quality parameters were very similar for all tests. Test temperatures for all 96-h tests were 24 ± 2^0 C. Dilution water salinity was 22 ± 2 ‰ for all tests. The D.O. ranged from 4.79 to 8.24 mg/l and the pH, from 7.7 to 8.6. Both of these physical parameters were within the acceptable limits for this test.

Mysid Assays

Analysis of mortality data (Table 1) indicated that *P. fumosoroseus* conidiospores were neither toxic nor pathogenic to mysids. Seawater control mortality was 5%. The highest mortality obtained was 20% for a conidiospore density of 1 X 10^5 per ml. This value was judged not to be significantly different from control mortality. Dead mysids showed no visible signs of fungal infection.

Shrimp feeding experiment

All shrimp appeared healthy with no visible signs of disease for the duration of the study. Each cube of the experimental diet contained viable *P. fumosoroseus* at a density of approximately 1 X 10^6 colony forming units. The agar formulation remained intact in the water. Shrimp held the cubes with their pleopods and fed vigorously. The dissolved oxygen in the aquaria ranged from 6.5 to 7.6 mg/l. At 3 weeks, *P. fumosoroseus* was cultured from fecal strands obtained from shrimp fed the experimental diet, but not from

fecal strands obtained from shrimp fed the heat-killed control diet. After 30 days, no mortalities were observed, and the experiment was terminated.

References

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Table 1. Mortality of *Mysidopsis bahia* (N=10) exposed to Conidiospores of *Paecilomyces fumosoroseus*

Count (conidiospores/ml)		Mortality (%)[a] following treatment with:	
Direct	Culturable	Viable conidiospores	Heat-killed conidiospores
1×10^4	9×10^3	0	ND
2×10^4	ND[b]	0	ND
1×10^5	9×10^4	20	ND
2×10^5	ND	0	ND
1×10^6	9×10^5	0	0
2×10^6	ND	10	10

[a]Seawater control mortality was 5%

[b]ND = not determined