Mycologia, 97(3), 2005, pp. 569–575.

Issued 16 September 2005

Factors influencing the sporulation and cyst formation of *Aphanomyces invadans*, etiological agent of ulcerative mycosis in Atlantic menhaden, *Brevoortia tyrannus*

Yasunari Kiryu[1]
Vicki S. Blazer

National Fish Health Research Laboratory, Biological Resources Division, U.S. Geological Survey, 11649 Leetown Road, Kearneysville, West Virginia 25430

Wolfgang K. Vogelbein
Howard Kator
Jeffrey D. Shields[2]

Department of Environmental and Aquatic Animal Health, Virginia Institute of Marine Science, Gloucester Point, Virginia 23062

Abstract: Oomycete infections caused by *Aphanomyces invadans* occur in freshwater and estuarine fishes around the world. Along the east coast of the USA, skin ulcers caused by *A. invadans* are prevalent in Atlantic menhaden, *Brevoortia tyrannus*. From laboratory observations low salinities appear crucial to transmission of the pathogen. To better understand aspects of transmission, we characterized sporulation and cyst formation of secondary zoospores of two isolates of *A. invadans* at different salinities and temperatures. Sporulation occurred only at low salinities. At room temperature (ca. 20–22 C), using ''pond water'' augmented with artificial sea salts, the endemic strain WIC and the Thailand strain PA7 of *A. invadans* produced free-swimming secondary zoospores at salinities of 0, 1 and 2 psu (practical salinity unit = ‰), but not at 4 psu or higher. Secondary zoospores of another species, ATCC-62427 (*Aphanomyces* sp.), were observed at 1, 2, 4 and 8 psu but not at 0 and 12 psu. Secondary zoospores of all three isolates, especially WIC, were abundant and motile 1–2 d postsporulation. Sporulation was temperature dependent and occurred over a relatively narrow range. No sporulation occurred at 4, 30 or 35 C for either WIC or PA7. For both strains zoospore production within 1–3 d after the initiation of sporulation was more prolific at 25 C than at 20 and 15 C. At 15 C production of zoospores was sustained over 11 d for WIC and 5 d for PA7. At room temperature single WIC secondary zoospores remained motile 12–18 h. Salinities exceeding 4 psu or vigorous shaking caused immediate cyst formation of WIC secondary zoospores. Exposure to menhaden tissue, but not tissues of other fishes to secondary zoospores (WIC), caused rapid (2 h) cyst formation. Cysts were capable of excysting when transferred to 1 psu water within 2–3 h of cyst formation. Cysts that had remained encysted in 6.5 psu for 24 h did not excyst when transferred to 1 psu water. Salinity and temperature requirements for sporulation indicate that juvenile menhaden must acquire infections during rain or in low salinity oligohaline waters.

Key words: epizootic ulcerative syndrome, fungal infections, *Pfiesteria,* salinity, temperature, zoospores

INTRODUCTION

Epizootic ulcerative syndrome (EUS) of fish is caused by the oomycete *Aphanomyces invadans* and is named variously ulcerative disease syndrome (UDS), ulcerative mycosis (UM), mycotic granulomatosis (MG), or red spot disease (RSD) (Lilley et al 1998). The syndrome has been reported worldwide and is widespread in aquaculture species in Southeast Asia (Roberts et al 1993), Japan (Egusa and Masuda 1971; Miyazaki and Egusa 1972, 1973a; Hatai et al 1977; Wada et al 1996) and Australia (Fraser et al 1992, Callinan et al 1989). The same or similar syndrome has been reported in Atlantic menhaden, *Brevoortia tyrannus,* along the east coast of the USA (Noga and Dykstra 1986, Blazer et al 1999). Kiryu et al (2002) fulfilled Koch's postulates by demonstrating that characteristic ulcerous skin lesions could be induced experimentally after inoculation or bath exposure with the secondary zoospores of an endemic isolate of *A. invadans.*

Aphanomyces invadans is an oomycete (order Saprolegniales) that can be maintained readily in laboratory culture in its hyphal stage (Lilley et al 1998). However this species lacks the usual sexual reproductive structures and is assigned to *Aphanomyces* on the basis of its pattern of asexual spore morphogenesis (Lilley et al 1998). When exposed to freshwater or water with low salinity, the hyphae produce sporangia and diplanetic zoospores are formed. The secondary zoospores eventually form secondary cysts, which germinate to form new hyphae. Cyst stages are infectious

Accepted for publication 3 Feb 2005.

[1] Current address: Fish and Wildlife Research Institute, Florida Fish and Wildlife Conservation Commission, 100 8th Ave. SE, St Petersburg, Florida 33701-5095.

[2] Corresponding author. E-mail: jeff@vims.edu

because they attach to the intact skin of menhaden and produce germination tubes that penetrate the skin (Kiryu et al 2003). However inoculated zoospores also can result in disease (Blazer et al 2002, Kiryu et al 2002), albeit they likely have to form cysts before penetrating tissue.

Sporulation of *A. invadans* has been examined previously but not in relation to ranges of temperature or salinity. Lilley et al (1998) used ''pond water'' as pond/lake water to support growth of *A. invadans* initiate sporulation. Griffin (1978) used an artificial sporulation medium (SM; 0.25 mM each of $CaCl_2$ and KCl) to generate spores of lower fungi. Fraser et al (1992) characterized *Aphanomyces* spp. and reported sporulation with SM and SM supplemented with 2 psu NaCl water but not at salinities higher than 4 psu. They evaluated hyphal growth at different temperatures, but sporulation was examined at room temperature. Given the nature of the infection in menhaden and the confusion over the etiology of ulcerous lesions in relation to *Pfiesteria* (e.g. Burkholder et al 2001), the effects of temperature and salinity on *A. invadans* sporulation needs to be examined to understand the ecology of infection and high prevalence of lesions observed in estuaries along the eastern coast of the USA.

Thus the objectives of this study were to: (i) examine sporulation of *A. invadans* in relation to different sources of pond water (i.e. natural sources of fresh reservoir, river and estuarine water); (ii) investigate the salinity and temperature range for sporulation in relation to transmission; (iii) determine the duration of zoospore production; and (iv) identify the factors influencing cyst formation and excystment of secondary zoospores.

MATERIAL AND METHODS

Fungal culture and sporulation.—Two isolates of *Aphanomyces invadans* were used in this study: (i) WIC, an endemic isolate from a menhaden with lesions captured in Maryland waters (USGS, Leetown, West Virginia; Blazer et al 1999) and (ii) PA7, an isolate from striped snakehead, *Channa striata,* from Nonthaburi, Thailand (Lilley and Roberts 1997). In addition a nonpathogenic isolate from menhaden from North Carolina also was examined. It formerly was identified as *A. invadans* but now is referred to as *Aphanomyces* sp., ATCC-62427 (American Type Culture Collection, Rockville, Maryland; Dykstra et al 1989, Blazer et al 2002). Isolates routinely were maintained on glucose peptone-penicillin-oxolinic acid agar (GP-POX agar; ingredients per liter reagent water were: 3 g glucose, 1 g peptone, 128 mg $MgSO_4 \cdot 7H_2O$, 14 mg $KH_2 \cdot PO_4$, 29 mg $CaCl_2 \cdot 2H_2O$, 2.4 mg $FeCl_3 \cdot 6H_2O$, 1.8 mg $MnCl_2 \cdot 4H_2O$, 3.9 mg $CuSO_4 \cdot 5H_2O$, 0.4 mg $ZnSO_4 \cdot 7H_2O$, 61 mg penicillin G, 10 mg oxolinic acid, and 12 g agar; Willoughby and Roberts 1994, Lilley et al 1998) for 4–5 d for WIC and PA7 or 2–3 d for ATCC 62427 followed by subculturing into GP-POX broth 3–4 wk at room temperature. For zoospore production, a piece of agar containing hyphae from the growing edge of a colony was aseptically excised and placed in 25 mL GP-POX broth in a 25 mL tissue-culture flask (Becton Dickinson Labware, Franklin Lakes, New Jersey). Cultures were allowed to grow 5–7 d before inducing sporulation. To induce sporulation cultures were washed twice with filtered (Whatman 54, Whatman International Ltd., Maidstone, England) and autoclaved estuarine water from the Great Wicomico River, Virginia, (adjusted to 1 psu) resuspended and incubated in the sterile water 12–24 h at room temperature. Zoospore densities were estimated by fixing a known volume of medium with 10% neutral-buffered formalin (4 : 1), centrifuging (10 min at 3500 × *g*), removing supernatant (1 : 10), resuspending cells to the starting volume and counting them in a hemacytometer (Neubauer/Bright-Line, Buffalo, New York).

Sporulation with different water sources.—The WIC isolate was tested against different water sources over a salinity range of 0–1 psu. Various fresh (FW) and estuarine water (EW) sources nearby the Virginia Institute of Marine Science (VIMS), Gloucester Point, Virginia, were tested: Beaver Dam Reservoir (FW), Poropotank River (FW), Great Wicomico River (EW) and Mattaponi River (EW). Water samples were held at room temperature several weeks with the cap of sample container loosely opened, then kept at 4 C until used. Two artificial sporulation media also were tested: sporulation medium (Griffin 1978; 0.25 mM each of $CaCl_2$ and KCl in deionized water [Nanopure]), and deionized water augmented with artificial sea salts (Marinemix Forty Fathoms, Marine Enterprises International Inc., Baltimore, Maryland) to 1 psu. All treatments were performed in triplicate. Densities of zoospores were estimated daily as described above or subjectively scored with a 10× objective and 10× ocular lenses on an Olympus IX50 inverted microscope with Hoffman modulation contrast (Tokyo, Japan). Densities were scored as: 0, zoospores not present; 1, 1–5 zoospores per field (low); 2, 6–20 zoospores per field (moderate); and 3, 21 or more zoospores per field (high).

Sporulation at different salinities.—Three isolates were tested: WIC, PA7 and ATCC-62427. Cultures were sporulated in triplicate at room temperature using water from Beaver Dam Reservoir augmented with artificial sea salts to 1, 2, 4, 8 and 12 psu. Secondary zoospore production was monitored daily for 6 d after initiating sporulation and subjectively scored for zoospore production with the scale described above.

Sporulation at different temperatures.—Two isolates were tested: WIC and PA7. Culture flasks were prepared routinely with GP-POX broth at the room temperature as described above. Triplicate cultures were sporulated in water from Beaver Dam Reservoir augmented with artificial seawater to 1 psu. Culture flasks were incubated at 4, 15, 20, 25, 30 and 35 C, and secondary zoospore production was scored daily with the subjective scale described above.

TABLE I. Secondary zoospore production of *Aphanomyces invadans* (WIC), an endemic strain isolated from the Wicomic River, Maryland

Water source	Salinity (psu)	Sporulation
Great Wicomico River	1	+
Beaver dam reservoir	0–1	+
Mattaponi River	1	+
Poropotank River	0–1	+
Sporulation media		−
Artifical seawater	1	−

Cyst formation and excystment at different salinities.—We used only the WIC isolate and water from the Poropotank River adjusted with artificial seawater to 1 psu. Poropotank River water was arbitrarily selected among the tested water sources because it previously had given consistent results. One day after initiating sporulation at room temperature, 1 mL volumes containing zoospores were distributed gently into 24-well tissue-culture plates to test the effects of salinity shock. After confirming zoospore motility with an inverted microscope, 1 mL of sterile artificial seawater with salinities of 1, 2, 4, 8 or 12 psu was added to each well (2 mL total volume) yielding final salinities of 1, 1.5, 2.5, 4.5 and 6.5 psu with four wells per treatment group. Zoospore motility was monitored periodically with an inverted microscope. After cyst formation, aliquots from each well were aspirated at 2, 4 and 24 h and washed twice with 1 psu water to observe for germination or zoospore activity.

Cyst formation with physical shock.—Secondary zoospores of the WIC strain generated from a 1 d old sporulation trial incubated at room temperature were placed in a 2 mL centrifuge tube and vigorously vortexed 10 s at maximum strength (Daigger Vortex, Scientific Industries Inc., Bohemia, New York). The suspension was transferred periodically into separate wells of a 24-well tissue-culture plate for observation. As a control, 0.5 mL of sporulated suspension was transferred gently to a well with a pipette.

Cyst formation and germination with fish mucus and tissues.—Fish mucus was collected with a cotton swab from a juvenile Atlantic menhaden captured from Sarah Creek, Virginia. The mucus was mixed gently into individual wells of a six-well tissue-culture plate containing 5 mL of a suspension of secondary zoospores. Mucus from live tilapia (*Oreochromis niloticus*), caudal fin from frozen menhaden (note that no obvious leaching of the blood occurred because only the fin clip was used) and live larval sheepshead minnows (*Cyprinodon variegatus,* 7–10 d old) also were assayed as described above.

RESULTS

Sporulation with different water sources.—All natural sources of water stimulated sporulation, while the artificial media did not (TABLE I). Secondary zoospores

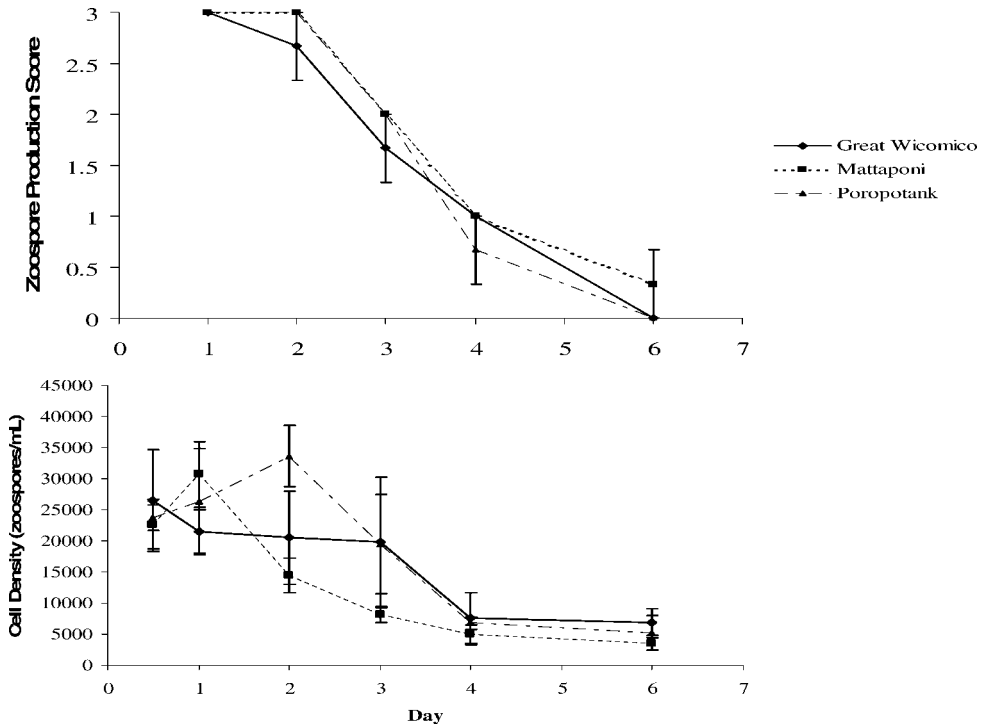

FIG. 1. Daily zoospore production, zoospore density (0–3 scale, upper panel) and estimated cell density (zoospore/mL, lower panel) of *A. invadans* (WIC strain) grown in different water sources (adjusted 1 psu) from Great Wicomico River, Mattaponi River and Poropotank River. Error bars = standard error of the mean.

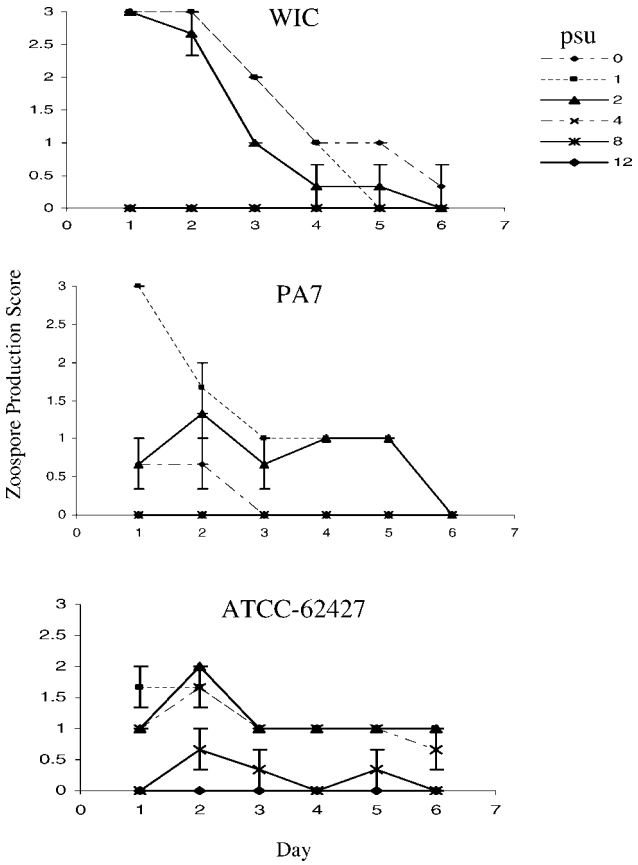

FIG. 2. Daily zoospore production (0–3 scale zoospore density) of *A. invadans* (WIC, PA7 and ATCC-62427 strains) at different salinities tested with Beaver Dam Reservoir water. Error bars = standard error of the mean.

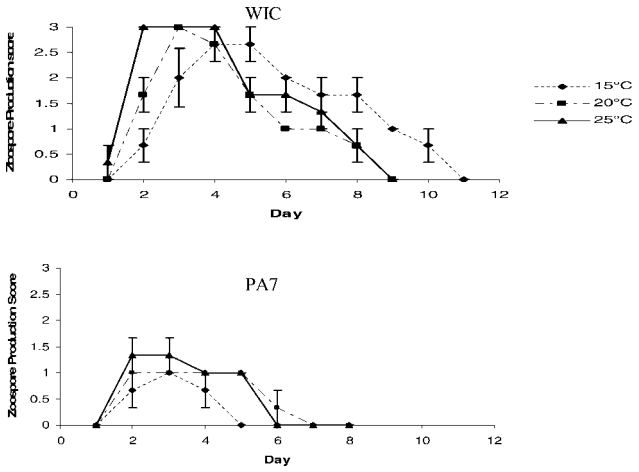

FIG. 3. Daily zoospore production (0–3 scale zoospore density) of *A. invadans* (WIC and PA7 strains) at different temperatures tested with Beaver Dam Reservoir water. Error bars = standard error of the mean.

appeared within 18–24 h after sporulation was initiated but production peaked in 1–2 d (FIG. 1). Peak densities were approximately 30 000 cells per mL and production declined markedly after Day 3. For the semiquantitative scale, 0 was equivalent to 0 zoospores observed in culture, 1 was equivalent to 1 to less than 5000 zoospores per mL, 2 was equivalent to 5000–20 000 zoospores per mL, and 3 was equivalent to greater than 20 000 zoospores per mL (FIG. 1). Secondary zoospores were observed in the media until 6 d. Hyphal colonies were no greater than 40 mm × 40 mm before production (data not shown).

Sporulation at different salinities.—Salinity affected sporulation of each isolate (FIG. 2). WIC and PA7 isolates exhibited zoospore production score at 0, 1 and 2 psu but not at 4 psu. For the first 1–2 d after initiation of sporulation, the WIC strain produced large numbers of zoospores at the three lowest salinities. The ATCC-62427 strain produced zoospores over a broader range from 1, 2, 4 and 8 psu but not at 0 or 12 psu. Zoospore production for all isolates continued approximately 6 d after initiation of sporulation.

Sporulation at different temperatures.—No sporulation was observed at 4, 30 or 35 C for either WIC or PA7 strains. Within the first 3 d of sporulation by the WIC strain, production of secondary zoospores was highest at 25 C, followed by 20 and 15 C (FIG. 3). At 15 C zoospore production lasted 11 d in WIC and 5 d in PA7. The mean score for zoospore production on a given day within 1–3 d after initiation of sporulation for PA7 was 1.3 (±0.33 SE) at 25 C compared to 3.0 (±0.0) at 20 and 25 C for WIC.

Cyst formation and excystment at different salinities.—Immediate cyst formation occurred when secondary zoospores (WIC) were transferred from 1 psu to 2.5, 4.5, 6.5 psu and higher salinities. Cysts were capable of excysting as secondary zoospores under limited conditions. Cysts generated by transferring into 6.5 psu environment and held 2–4 h were able to excyst gradually as secondary zoospores again when placed in 1 psu water. Cysts held 24 h at 6.5 psu then transferred to 1 psu water did not excyst. After 24 h several of these cysts had germinated.

Cyst formation with physical shock.—Isolated zoospores formed cysts naturally after 12–18 h in 1 psu water. However, physical shock caused immediate cyst formation.

Cyst formation and germination with fish mucus and tissues.—Cyst formation also was observed after contact of zoospores with mucus obtained from menhaden but not from tilapia or larval sheepshead minnows. Zoospores exhibited pronounced behavioral changes when in contact with mucus from

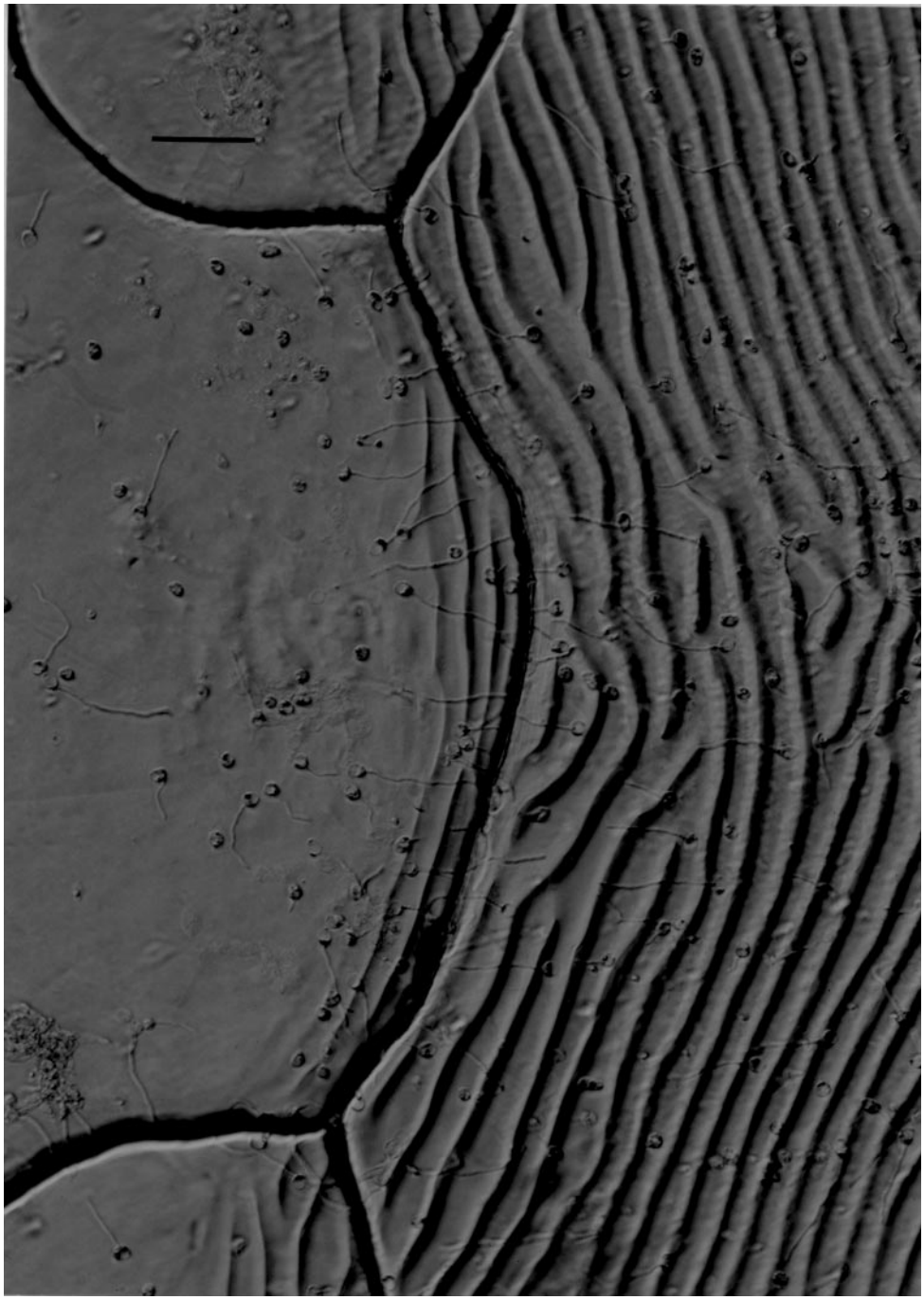

FIG. 4. Photomicrograph of secondary zoospores encysted on a piece of Atlantic menhaden scale and producing germination tube. Hoffman modulation contrast; bar = 50 μm; total magnification = 290×.

menhaden. Secondary zoospores normally moved randomly through the medium, but upon contact with menhaden mucus they swam in slow arcs with motion directed toward the mucus or swam in a circular fashion. The same behavior was observed when a menhaden caudal fin was introduced to a suspension of secondary zoospores. In addition several (10–20) zoospores typically formed cysts around scales that had fallen from the caudal peduncle (FIG. 4). Germination was seen within 2–6 h of exposure to menhaden mucus or fin. Altered zoospore swimming patterns were not observed in the unexposed control treatment or in treatments of zoospores exposed to tilapia mucus or larval minnows. In addition scanning electron microscopy observation of sheepshead minnow skin after exposure

to zoospores for 24 h confirmed no attachment to the skin.

DISCUSSION

In the natural environment, menhaden must acquire infections in waters with low salinity or presumably in association with heavy rainfall, during which surface salinities are reduced to 2 psu or lower (Kator et al 2000). In the Tar-Pamlico rivers estuary, North Carolina, Levine et al (1990a) found higher prevalence of menhaden infected with ulcerative mycosis (UM) at lower salinities (2.5–9.6 psu) compared to higher salinities (13.5–15.7 psu). Salinity in the open ocean varies between 33–35 psu. Virgona (1992) conjectured that rainfall might play a significant role in the prevalence and onset of lesions because the prevalence of lesions was higher during years with greater rainfall. Our findings support the hypothesis that transmission must occur in water with low salinity or in waters overlying the estuarine high salinity wedge. Given the large quantity of zoospores produced by a colony of the oomycete, we suggest that whole schools of menhaden are exposed to low salinity during runoff, which accounts for the prevalence of infection observed in the field. Atlantic menhaden often school in low salinity reaches of estuaries in mid-Atlantic states, particularly near the chlorophyll maxima (Friedland et al 1996). Thus they obtain infections while swimming through low salinity while maturing in these estuaries.

Sporulation of *Aphanomyces invadans* requires a narrow salinity window. The WIC and PA7 strains sporulated only between 0 and 2 psu. The nonpathogenic ATCC-62427 isolate was more halotolerant and sporulated between 1 and 8 psu but not at 0 or 12 psu. Two of the strains tested in this study, WIC and PA7, are the same species as confirmed by polymerase chain reaction analysis targeting internal transcribed spacer region between 18S and 5.8S rRNA gene (Blazer et al 2002). The two strains appear to have somewhat different characteristics with regard to sporulation; WIC produced more zoospores than PA7 and it produced them over a more sustained period between 15 and 25 C. Prolonged in vitro culture conditions or adaptation to cooler temperate climates could cause these differences. Yet the sporulation patterns of both strains as a function of temperatures were similar: sporulation between 15 and 25 C, no sporulation at 4 C or above 30 C. PCR confirmed the ATCC-62427 isolate is not *A. invadans* (Blazer et al 2002) and is not pathogenic to Atlantic menhaden (Kiryu et al 2002); it might be a halotolerant saprophyte arising from the initial isolation.

Sporulation of *Aphanomyces invadans* was observed in all the natural water samples tested in this study (Lilley et al 1998) but was not observed in pure laboratory water or SM water, suggesting that some unknown factor or factors are necessary for the sporulation of this organism.

Menhaden mucus and tissues served as potential cues for cyst formation of zoospores and germination of cysts. The fact that no response was seen with mucus or tissues of tilapia or larval sheepshead minnow is intriguing and suggests a more refined relationship between the oomycete and Atlantic menhaden. In Asia *A. invadans* infects a broad range of host species such as snakehead, *Channa striata* (Roberts et al 1993); ayu, *Plecoglossus altivelis* (Egusa and Masuda 1971, Miyazaki and Egusa 1973a, Hatai et al 1977); and goldfish, *Carassius auratus* (Miyazaki and Egusa 1972). However several species including tilapia are resistant to *A. invadans,* and this resistance is based primarily on an intense cellular immunity (Kahn et al 1998). In the USA, several species of Atlantic coast fishes recently have been shown to be susceptible to the oomycete via inoculation (Johnson et al 2004), but only menhaden show a prevalence of lesions in the field (Levine et al 1990a). Our data support that infectivity is largely a function of specific host recognition factors on the surface of susceptible fishes.

ACKOWLEDGMENTS

This research was financed in part by ECOHAB Grant R-828225, the U.S. Geological Survey Chesapeake Bay Initiative and the Virginia Institute of Marine Science's *Pfiesteria* Initiative. We thank Dave Zwerner, Patricia Blake, Patrice Mason, Kyle Hayes, RaeMarie Johnson, Jessica Tiedeken, Christopher Squyars, Alynda Miller, Dana Booth and Martha Rhodes at VIMS. This is Virginia Institute of Marine Science contribution 2688.

LITERATURE CITED

Blazer VS, Lilley J, Schill WB, Kiryu Y, Densmore CL, Panyawachira V, Chinabut S. 2002. *Aphanomyces invadans* in Atlantic menhaden along the east coast of the United States. J Aqua An Heal 14:1–10.

———, Vogelbein WK, Densmore CL, May EB, Lilley JH, Zwerner DE. 1999. *Aphanomyces* as a cause of ulcerative skin lesions of menhaden from Chesapeake Bay tributaries. J Aqua An Heal 11:340–349.

Burkholder JM, Glasgow HB, Deamer-Melia N. 2001. Overview and present status of the toxic *Pfiesteria* complex (Dinophyceae). Phycologia 40:186–214.

Callinan RB, Fraser GC, Virgona JL. 1989. Pathology of red spot disease in sea mullet, *Mugil cephallus* L., from eastern Australia. J Fish Dis 12:467–479.

Dykstra MJ, Levine JF, Noga EJ, Hawkins JH, Gerdes P, Hargis Jr WH, Grier HJ, Te Strake D. 1989. Ulcerative mycosis: a serious menhaden disease of the southeastern

coastal fisheries of the United States. J Fish Dis 12:175–178.

Egusa S, Masuda N. 1971. A new fungal disease of *Plecoglossus altivelis*. Fish Path 6:41–46 (In Japanese).

Fraser GC, Callinan RB, Calder LM. 1992. *Aphanomyces* species associated with red spot disease: an ulcerative disease of estuarine fish from eastern Australia. J Fish Dis 15:173–181.

Friedland KD, Ahrenholz DW, Guthrie JF. 1996. Formation and seasonal evolution of Atlantic menhaden juvenile nurseries in coastal estuaries. Estuaries 19:105–114.

Griffin DH. 1978. *Achlya bisexualis*. In: Fuller MS, ed. Lower fungi in the laboratory. University of Georgia, Athens. p 67–68.

Hatai K, Egusa S, Takahashi S, Ooe K. 1977. Study on the pathogenic fungus of mycotic granulomatosis. I. Isolations and pathogenecity of the fungus from cultured ayu infected with the disease. J Fish Path 12:129–133 (In Japanese).

Johnson RA, Zabrecky J, Kiryu Y, Shields JD. 2004. Infection experiments with *Aphanomyces invadans* in four species of estuarine fishes. J Fish Dis 27:287–295.

Kator H, Haas LW, Vogelbein WK, Zwerner DE, Shields JD. 2000. An integrated field study to identify factors responsible for ulcerative lesions in juvenile menhaden in the Great Wicomico river, Virginia. Symposium on Harmful Marine Algae in the U.S. Marine Biological Laboratory, Woods Hole, Massachusetts, 4–9 Dec 2000.

Khan MH, Marshall L, Thompson KD, Campbell RE, Lilley JH. 1998. Susceptibility of five fish species (Nile tilapia, rosy barb, rainbow trout, stickleback and roach) to intramuscular injection with the oomycete fish pathogen, *Aphanomyces invadans*. Bull Euro Assoc Fish Path 18: 192–197.

Kiryu Y, Shields JD, Vogelbein WK, Zwerner DE, Kator H, Blazer VS. 2002. Induction of skin ulcers in Atlantic menhaden by injection and aqueous exposure to the zoospores of *Aphanomyces invadans*. J Aqua An Heal 14:11–24.

———, ———, ———, Kator H, Blazer VS. 2003. Infectivity and pathogenicity of the oomycete *Aphanomyces invadans* in Atlantic menhaden *Brevoortia tyrannus*. Dis Aqua Organ 54:135–146.

Levine JF, Hawkins JH, Dykstra MJ, Noga EJ, Moye DW, Cone RS. 1990a. Species distribution of ulcerative lesions on finfish in the Tar-Pamlico River Estuary, North Carolina. Dis Aqua Organ 8:1–5.

———, ———, ———, ———, ———, ———. 1990b. Epidemiology of ulcerative mycosis in Atlantic menhaden in the Tar-Pamilico esuary, North Carolina. J Aqua An Heal 2:162–171.

Lilley JH, Callinan RB, Chinabut S, Kanchanakhan S, MacRae IH, Phillips MJ. 1998. Epizootic ulcerative syndrome (EUS) technical handbook. The Aquatic Animal Health Research Institute, Bangkok, Thailand.

———, Roberts RJ. 1997. Pathogenecity and culture studies comparing the *Aphanomyces* involved in epizootic ulcerative syndrome (EUS) with other similar fungi. J Fish Dis 20:135–144.

Miyazaki T, Egusa S. 1972. Studies on mycotic granulomatosis in fresh water fishes—I. Mycotic granulomatosis in goldfish. Fish Path 7:15–25 (In Japanese).

———, ———. 1973a. Studies on mycotic granulomatosis in fresh water fishes—II. Mycotic granulomatosis in Ayu, *Plecoglossus*. Fish Path 7:125–133 (In Japanese).

———, ———. 1973b. Studies on mycotic granulomatosis in fresh water fishes—III. Mycotic granulomatosis in blue-gill. Fish Path 8:41–43 (In Japanese).

———, ———. 1973c. Studies on mycotic granulomatosis in fresh water fishes—IV. Mycotic granulomatosis in wild fishes. Fish Path 8:44–47 (In Japanese).

Noga EJ, Dykstra MJ. 1986. Oomycete fungi associated with ulcerative mycosis in menhaden, *Brevoortia tyrannus* (Latrobe). J Fish Dis 9:47–53.

Roberts RJ, Willoughby LG, Chiabut S. 1993. Mycotic aspects of epizootic ulcerative syndrome (EUS) of Asian fishes. J Fish Dis 16:169–183.

Virgona JL. 1992. Environmental factors influencing the prevalence of a cutaneous ulcerative disease (red spot) in the sea mullet, *Mugil cephalus* L., in the Clarence River, New South Wales, Australia. J Fish Dis 15:363–378.

Wada S, Rha S, Kondoh T, Suda H, Hatai K, Ishi H. 1996. Histopathological comparison between ayu and carp artificially infected with *Apahnomyces piscicida*. Fish Path 31:71–80.

Willoughby LG, Roberts RJ. 1994. Improved methodology for isolation of the *Aphanomyces* fungal pathogen of epizootic ulcerative syndrome (EUS) in Asian fish. J Fish Dis 17: 541–543.