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Aphanomyces frigidophilus sp. nov. from eggs of Japanese char, *Salvelinus leucomaenis*

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***Aphanomyces frigidophilus* sp. nov. was obtained from eggs of Japanese char, *Salvelinus leucomaenis*, from Tochigi Prefectural Fisheries Experimental Station, Utsunomiya, Japan. Vegetative hyphae were delicate, slightly wavy, moderately branched. Zoosporangia were isodiametric with the vegetative hyphae. Oogonia were abundant, originating on short stalks from lateral sides of hyphae. Oogonia were spherical, subspherical or pyriform, with a single subcentric oospore inside. Outer surfaces of oogonia were roughened with short papillate, crenulate or irregular ornaments. Antheridia and oospore germination were not observed. Zoospore germination and vegetative growth were found from pH 5.0 to 11.0. Zoospore production was highest at 10°C, whereas rapid growth occurred at 20–25°C. Vegetative growth of the fungus declined from the maximal level at 25°C to less than half maximal at 30°C and completely disappeared at 35°C.**

Key Words——*Aphanomyces frigidophilus*; morphology; pH; temperature.

Several reports on fungal species associated with eggs of various fish species (Srivastava and Srivastava, 1976a,b; Czeczuga and Woronowicz, 1993; Diler, 1995) indicate that fungi in the family Saprolegniaceae are one of the major groups involved and that infection by *Aphanomyces* is rare compared with that by *Saprolegnia*. However, fungi in the genus *Aphanomyces* have been occasionally reported on fish eggs. *Aphanomyces laevis* is the only species of this genus that has been reported on eggs of rainbow trout, *Oncorhynchus mykiss* Walbaum (Scott and O'Bier, 1962), and vendace, *Coregonus albula* L. (Czeczuga and Woronowicz, 1993), although an unidentified *Aphanomyces* was also reported on rainbow trout (Scott and O'Bier, 1962).

This study was carried out on a fungus isolated from the eggs of Japanese char, *Salvelinus leucomaenis* (Pallas). The fungus was classified in the genus *Aphanomyces*, subgenus *Axyromyces*, and was found to differ from the other two species in this subgenus. Morphological details and biological characteristics of the fungus are described.

Materials and Methods

Isolation and identification Eggs of Japanese char kept in a 50 L plastic vat with running water at approximately 9°C at Tochigi Prefectural Fisheries Experimental Station, Utsunomiya, Japan, became overwhelmed with fungal mycelia. Eggs whose outer membranes were obviously infected with fungus were taken and successively washed with sterilized well water. The membranes were then placed in 25 ml of fresh sterilized well water and kept at 15°C for 24 h. The zoospore suspension thus obtained was diluted with sterilized well water to a concentration of 100–200 spores/ml. Streptomycin sulfate and ampicillin at concentrations of 150 and 100 μg/ml, respectively, were added to the suspension to obtain bacteria-free cultures. Then 0.5 ml of zoospore suspension was spread on GY agar plates, prepared as described by Kitancharoen et al. (1995), and incubated at 15°C for 18–24 h. Four or five replicates were made. Single young germinating thalli were observed under an inverted microscope and transferred to separate GY agar plates. The isolates were held on GY agar and subcultures were taken every 3–4 wk. Observation of the zoospore-release pattern in sterilized well water revealed that all isolates belonged to the genus *Aphanomyces*. Further research into temperature response and sexual reproduction confirmed that all isolates were homogeneous. Therefore, isolate NJM 9500 was selected as a representative isolate for further study. Sexual reproduction was induced using hemp seed culture at 10°C. The fungal isolate was identified by reference to the works of Scott (1961), Hatai (1980) and Willoughby et al. (1995).

Effect of pH on vegetative growth GY broth was autoclaved, its pH was adjusted to various values in the range of 3.5–11.0 with 5 N NaOH or concentrated HCl, then the broth was filtered through 0.2 μm Millipore filter paper (Whatman). Nine agar plugs with mycelia of a 2 d-cultured colony of isolate NJM 9500 on GY agar were cut out with a no. 1 cork borer (4.5 mm in diam), placed into 10 ml of the GY broth and held at 20°C for 3 d, then the colony diameter at each pH was measured.

Effect of pH on zoospore germination A zoospore suspension was obtained by inoculating an agar block (about 5×5 mm) with the actively growing mycelia of isolate NJM 9500 into a small disposable Petri dish (50×15 mm) containing 10 ml of GY broth and held at 20°C for 48 h. Mycelia were removed and washed repeatedly in sterilized well water, then transferred to 10 ml of fresh sterilized tap water and kept at 10°C for 24 h. The resulting zoospore suspension was adjusted to a concentration of 1×10^4 spores/ml. Then 100 μl of zoospore suspension was inoculated into small disposable Petri dishes containing 10 ml of GY broth adjusted to various pHs in the range of 3.5–11.0 as described previously. The inoculated plates were incubated at 20°C for

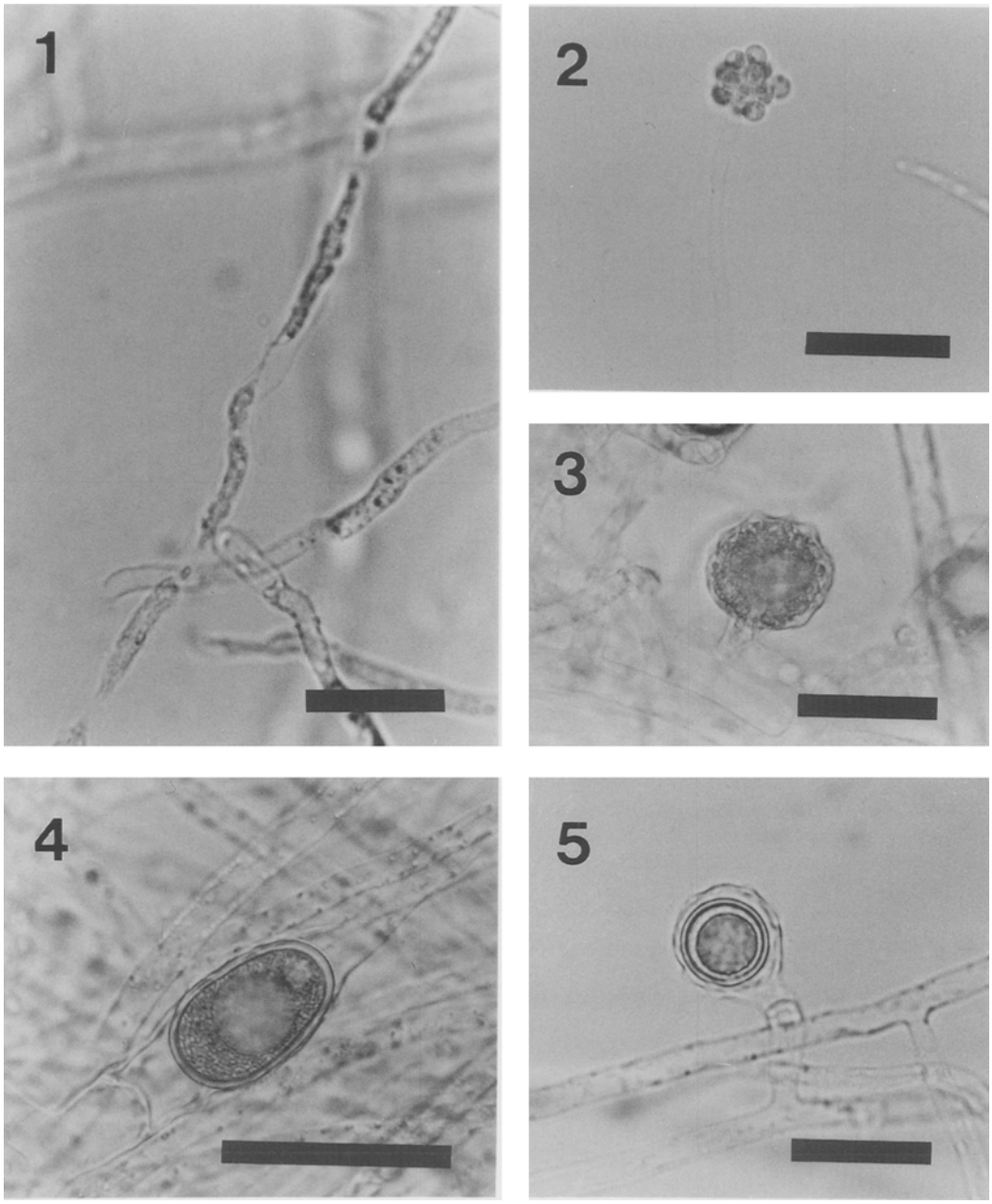

Fig. 1. Primary zoospore of *Aphanomyces frigidophilus* formed in hypha as a single row of elongated tapering spores connected by fine protoplasmic threads. Scale bar=30 μm.
Fig. 2. Primary zoospores emerged and encysted at the orifice in a cluster. Scale bar=60 μm.
Fig. 3. Young oogonium of *A. frigidophilus* with roughened outer contour. Scale bar=30 μm.
Fig. 4. Elongated oogonium formed between vegetative hypha. Scale bar=50 μm.
Fig. 5. Mature oogonium of *A. frigidophilus* showing single, subcentric oospore inside without attached antheridium. Scale bar=30 μm.

3 d to observe zoospore germination.

Effect of temperature on vegetative growth Isolate NJM 9500 was used. The advancing edges of a 2 d-cultured colony on GY agar were cut out with a no. 1 cork borer and placed on the center of 90 × 20 mm disposable Petri dishes containing 20 ml of GY agar, then incubated at 5, 10, 15, 20, 25, 30 or 35°C. Colony radial growth from the blocks was measured and expressed as the mean of four perpendicular radii.

Effect of temperature on zoospore production Mycelia of the fungal isolate on excised GY agar blocks were grown in GY broth at 20°C for 48 h (hyphae extended from the agar block about 1 cm), then removed and washed with sterilized tap water. Approximately equal amounts of mycelia were then placed into small Petri dishes with 10 ml of sterilized tap water and held at 10, 20 or 30°C. The number of swimming zoospores was observed and counted using Neubauer counting chamber (Erma^{R}) every day for 1 wk, following which the occurrence of swimming zoospores was checked once every week for 1 mo.

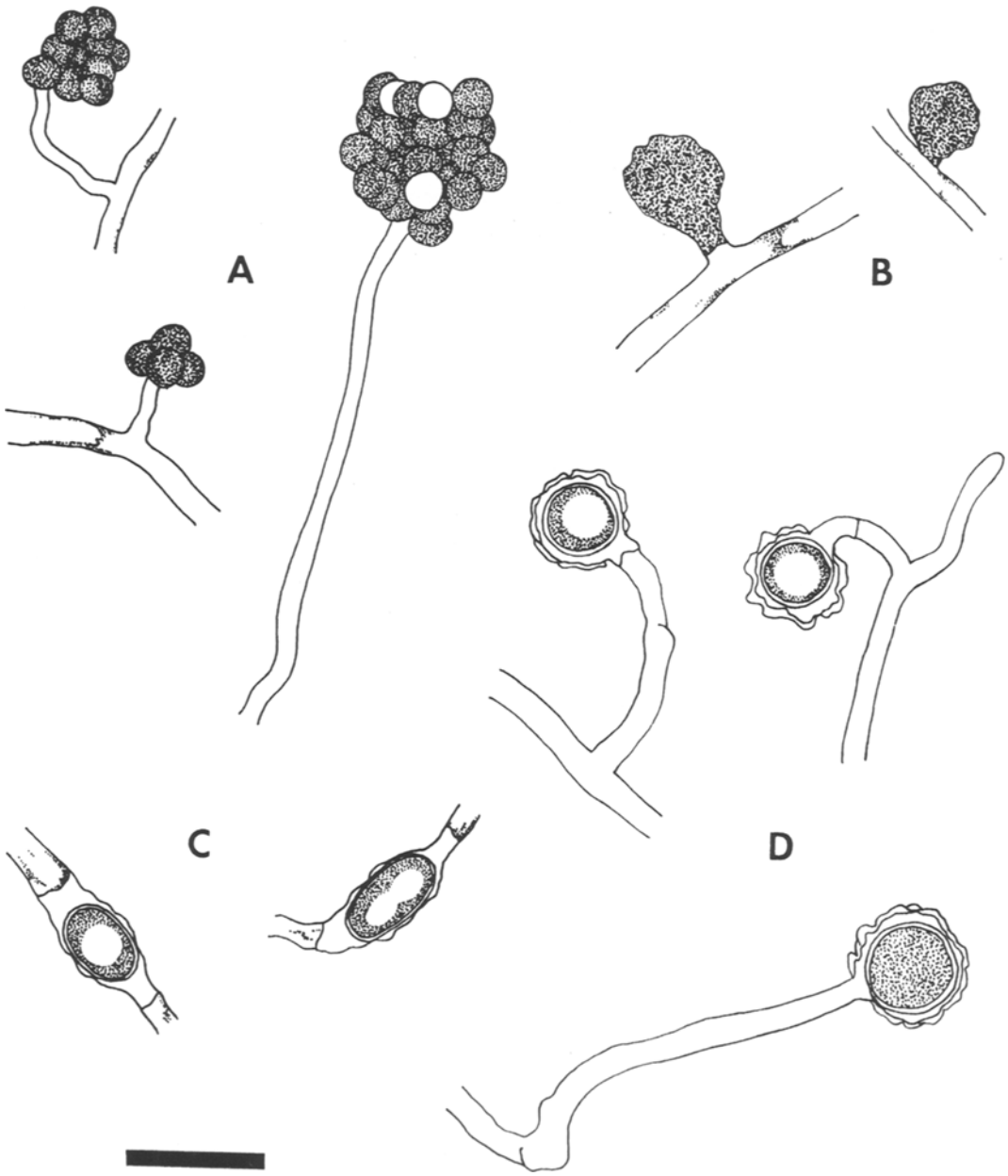

Fig. 6. *Aphanomyces frigidophilus*.
A. Clusters of encysted spores with some empty cysts remaining after secondary zoospore release; B. Young oogonia with roughened outer contours; C. Elongated oogonia between hyphae; D. Oogonia in different stages of oospore development: above filled with matured oospores and below filled with immature oospores, the outer contours were short papillate or irregular. Scale bar = 30 μm.

Results

Identification The isolate NJM 9500 was apparently not identical with any member of *Aphanomyces* hitherto described. In the monograph of Scott (1961), no *Aphanomyces* spp. without antheridium are reported. *Aphanomyces piscicida* Hatai (1980) and *A. invaderis* Willoughby et al. (1995), both displayed no sexual stage. Consequently the following name was proposed for the isolate NJM 9500:

Aphanomyces frigidophilus Kitancharoen & Hatai, sp. nov. Figs. 1–6

Mycelium aseptatum, subtile, 7–10 μm diam, laeve, leviter undulatum, modice ramosum; zoosporangia isodiametra diametrum hyphae aequantia; zoosporae prope orificio emergentes et incystatae, conglobatae in globum; zoosporae secundae reniformes, latere biflagellatae; oogonia copiosa, breviter stipitata, pyriformia, sphaerica vel subsphaerica, 16–25 μm diam, extere breviter papillato, crenulato vel irregulariter eminenti praedita, interdum elongata et hyphas intercalaria; oosporae singulares, generatim sphaericae, 14-22 μm diam, subcentricae; antheridium nullum.

Holotypus: NJM 9500, colonia exsiccata e cultura ex ovo *Salvelini leucomaenis* (Pallas) in Utsunomiya, Tochigi Pref., Japonia, XII-1995, a N. Kitancharoen isolata et ea collectione culturae in Universitate Veterinarii et Scientificae Animalis Nipponensis (NJM) conservata.

Aphanomyces frigidophilus, registered as NJM 9500, isolated from the eggs of Japanese char, *Salvelinus leucomaenis*, in Tochigi Prefecture in December 1995. Vegetative mycelium was delicate, about 7–10 μm in diam, aseptate, smooth, slightly wavy, moderately branched. Zoosporangia were isodiametric, considerably long; cross-walls were seldom observed. Zoospores formed by segregation of protoplasm into a single row of elongated tapering spores, of which most were connected by fine protoplasmic threads (Fig. 1). Primary zoospores near the orifice emerged in this manner, whereas those emerging later became elongate with rounded ends during the passage through zoosporagium. Upon emergence, spores were irregularly shaped, but immediately assumed a spherical shape and encysted in cluster at the orifice (Figs. 2, 6A). Secondary zoospores were reniform, laterally biflagellate. Oogonia were abundant on a short oogonial stalk, usually pyriform, spherical or subspherical, 16–25 μm in diam, with a short papillate, crenulate or irregular outer contour, even in young oogonia (Figs. 3, 6B). Oogonia were occasionally elongate, intercalary in hyphae (Figs. 4, 6C). A single oospore mostly filled the oogonium and was dominantly spherical, 14–22 μm in diam, occasionally obovate or irregular depending on oogonial shape. Oospores were dominantly subcentric with a large shiny vesicle surrounded by fine granules (Figs. 5, 6D). No antheridium was detected in the sexual stage of the isolate.

Effect of pH on vegetative growth and zoospore germination Vegetative growth of *A. frigidophilus* appeared in GY broth in the pH range of 5.0–11.0. The pH range of 7.0–9.0 appeared to be optimum for hyphal growth. Hyphal growth declined at pH 10.0. Also, zoospore germination was discovered in GY broth in the pH range of 5.0–11.0 (Fig. 7).

Effect of temperature on vegetative growth and zoospore production As shown in Fig. 8, the fungus was able to grow in the temperature range of 5–30°C, with maximal growth at 25°C. Zoospore production, on the other hand, was more active at low temperatures, especially at 10°C. Zoospore production decreased as the tempera-

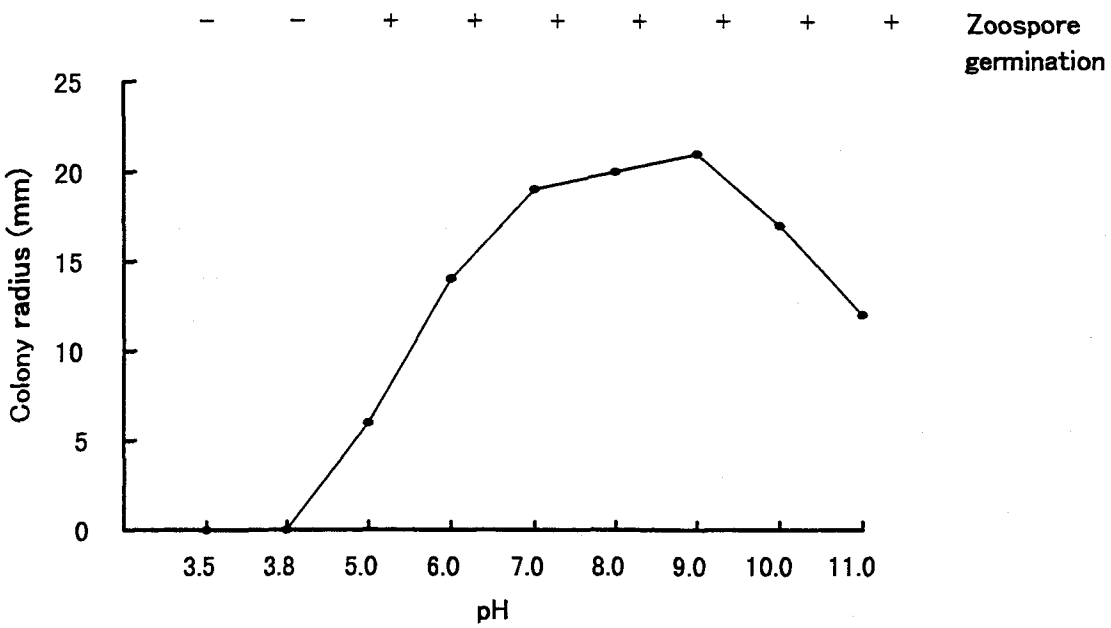

Fig. 7. Effect of pH on vegetative growth and zoospore germination (+: germinated, −: not germinated) of *Aphanomyces frigidophilus* after incubation at 20°C for 2 d.

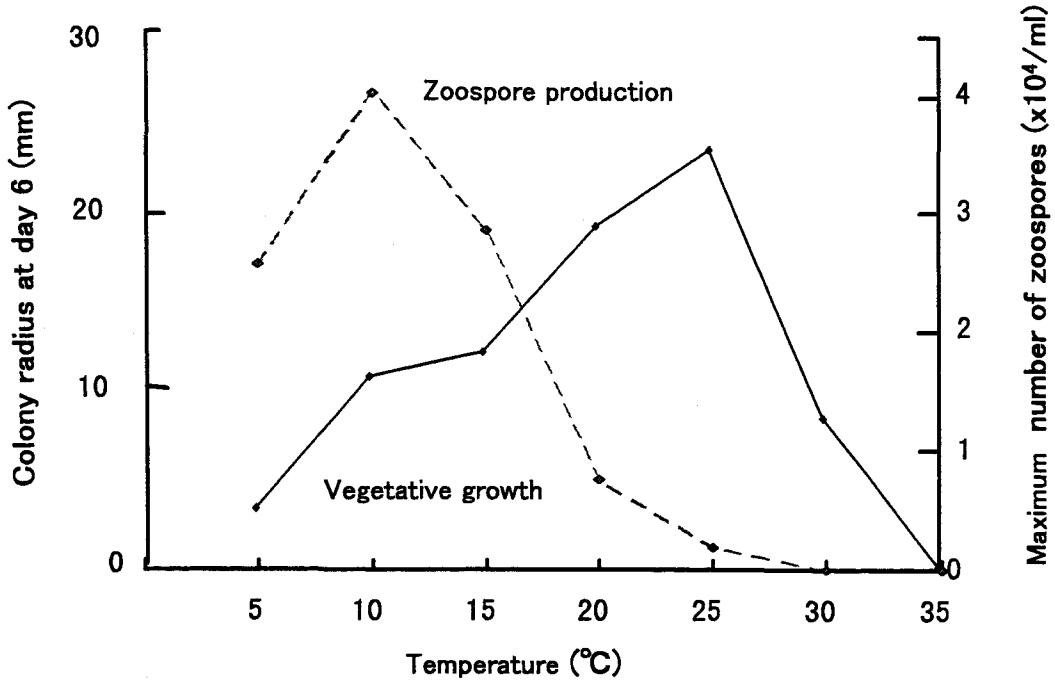

Fig. 8. Effect of temperature on vegetative growth and zoospore production of *Aphanomyces frigidophilus*.

ture increased and completely vanished at 30°C.

Discussion

Aphanomyces frigidophilus was classified in the subgenus *Axyromyces* based on the appearance of oogonia with roughened outer contours without definite spines or tubercles, according to Scott (1961). There are two species in this subgenus, *A. ovidestruens* Gicklhorn and *A. irregulare* Scott. *Aphanomyces ovidestruens* is distinguished by oogonia with a smooth contour which becomes roughened at maturity. These obviously differed from *A. frigidophilus*, in which even young oogonia had roughened contours. In comparison with *A. irregulare*, the other similar species, *A. frigidophilus* apparently differs in the antheridial appearance. *Aphanomyces irregulare* seems to have diclinous or monoclinous antheridia which are sometimes absent (Scott, 1961), whereas *A. frigidophilus* displayed no antheridium attached to oogonia. Additionally, neither *A. ovidestruens* nor *A. irregulare* has hitherto been reported on fish eggs.

Aphanomyces frigidophilus could grow in acidic to alkaline conditions, but it seems likely that the fungus preferred neutral to weak alkaline conditions, pH 7.0–9.0, since it grew best in this pH range. Zoospore germination was found in the same pH range as vegetative growth. Kitancharoen et al. (1996) reported that *Saprolegnia diclina* Humphrey and *S. parasitica* Coker were able to grow and germinate even in strongly acidic conditions such as pH 3.5 or 3.8. With regard to temperature, the fungus was considered to favor producing zoospores at low temperature, since zoospore production declined when the temperature increased. Dieguez-Uribeondo et al. (1994) proposed that zoospores are primarily involved with parasitic activity of the Oomycetes. The fact that vegetative growth of *A. frigidophilus* was highest at 25°C suggests that high rates of metabolic activity were still under control at this temperature. Since vegetative growth declined to less than half maximal at 30°C and completely disappeared at 35°C, the maximum temperature threshold for normal growth of this fungus appeared to be 25°C.

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