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Lagenidium myophilum infection in the coonstripe shrimp, *Pandalus hypsinotus*

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A fungal infection occurred in juvenile coonstripe shrimps, *Pandalus hypsinotus*, cultured at Hokkaido Institute of Mariculture, Hokkaido, Japan. The fungus was identified as *Lagenidium myophilum*, the same fungus that had previously been isolated from the abdominal muscle of adult northern shrimps, *Pandalus borealis*, and larvae of the coonstripe shrimp. Histopathologically, numerous nonseptate hyphae were observed in the lesions, and melanized hemocytes were present within the blackened areas. The optimum temperature for growth of the present strain was 25-30°C, and the optimum NaCl concentration for growth was 0.5-1.0%. Its biological characteristics were compared with those of *Lagenidium myophilum* isolated from diseased larval coonstripe shrimp and adult northern shrimp. The fungus was pathogenic toward shrimps of the genus *Pandalus*, which live in deep sea areas. The fungus could infect shrimps at various stages, from larva to adult.

Key Words——coonstripe shrimp; fungal infection; *Lagenidium myophilum*; *Pandalus hypsinotus*.

Members of the genus *Lagenidium* are known as parasites of algae, fungi belonging to Mastigomycotina, and microscopical animals (Sparrow, 1960; Sparks, 1985). Infection by *Lagenidium myophilum* Hatai & Lawhavinit, found in the abdominal muscle of adult northern shrimp, *Pandalus borealis* Krøyer, has only been reported in Japan (Hatai and Lawhavinit, 1988). In the genus *Lagenidium, L. callinectes* Couch has been isolated from eggs of the blue crab, *Callinectes sapidus* Rathbun (Couch, 1942), and the barnacle, *Chelonibia patula* Ranzani (Johnson and Bonner, 1960); *L. scyllae* Bian et al. from eggs and larvae of the mangrove crab, *Scylla serrata* Forskål (Bian et al, 1979); *L. chthamalophilum* Johnson from eggs of the barnacle, *Chthamalus fragilis* Darwin (Johnson, 1958); all have previously been reported as parasites of crustaceans. In addition, infection by species of *Lagenidium* has been reported in larvae of the white shrimp, *Penaeus seriferus* Linnaeus (Lightner and Fontain, 1973), the Dungeness crab, *Cancer magister* Dana (Armstrong et al, 1976), and the American lobster, *Homarus americanus* Milne-Edwards (Nilson et al, 1976).

In 1991, *L. myophilum* infection occurred in larvae of coonstripe shrimps, artificially produced at Hokkaido Institute of Mariculture, Hokkaido. Mortality was 100% (Hatai, unpublished). In 1993, a fungal infection occurred in juvenile coonstripe shrimps which had been reared in tanks after seed production. Mortality was about 70%.

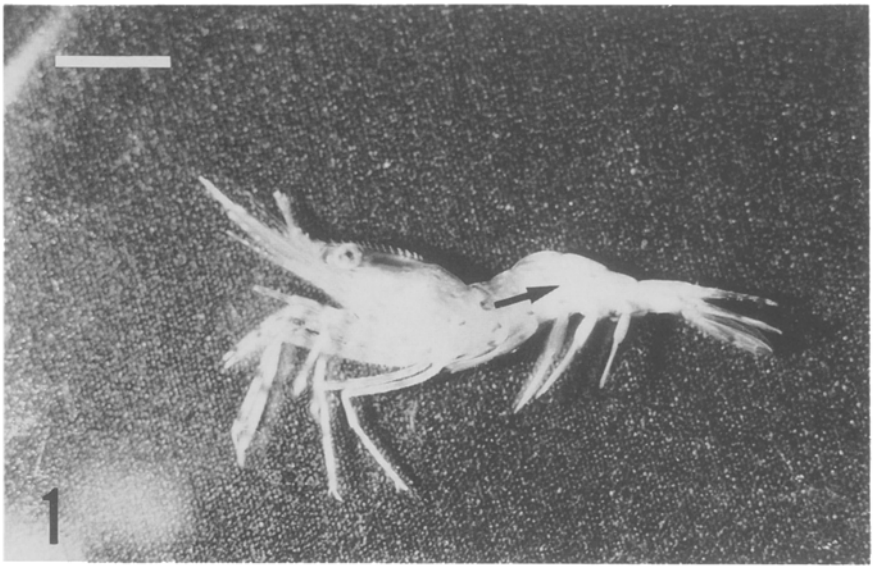
This study describes the morphological and physiological characteristics of the fungus isolated from the lesions, and compares the present strain with two strains of *L. myophilum*, NJM 9131 and NJM 8601, isolated from the larvae of the coonstripe shrimp in 1991 and the abdominal muscle of adult northern shrimp in 1986, respectively.

Materials and Methods

Isolations Juvenile coonstripe shrimps dying from fungal infection were obtained from the Hokkaido Institute of Mariculture, Hokkaido on 10 January 1993. The shrimps measured 55-89 mm (av. 72.6 mm) carapace length, and 20-50 g (av. 29.2 g) body weight. These shrimps were cultivated at 9°C. The diseased juvenile coonstripe shrimps, which were hatched from adult shrimps collected from deep sections of Sea of Japan and Uchiura Wan were reared in tanks. Lesions were found on the muscle and characterized mainly by a whitish or sometimes black color (Fig. 1). Many nonseptate hypae measuring approximately 3-8 μm diam were found in the lesions by direct microscopical examination (Fig. 2).

Numerous oil globules and vacuoles were observed in the hyphae. Parts of the lesions were inoculated onto PYGS agar to isolate the fungus. Small amounts of streptomycin sulfate and ampicillin were added to the medium to reduce bacterial growth. The plates were incubated at 10°C for 10 days. After fungal growth was observed, a 0.5-cm-square block of agar with mycelium was cut out and transferred onto the same medium to make a pure culture. A typical isolated strain, NJM 9331, was used for all experiments.

Histopathology The infected shrimps were fixed in a 10% (v/v) phosphate buffered formalin solution, then transferred into formic acid for decalcification and



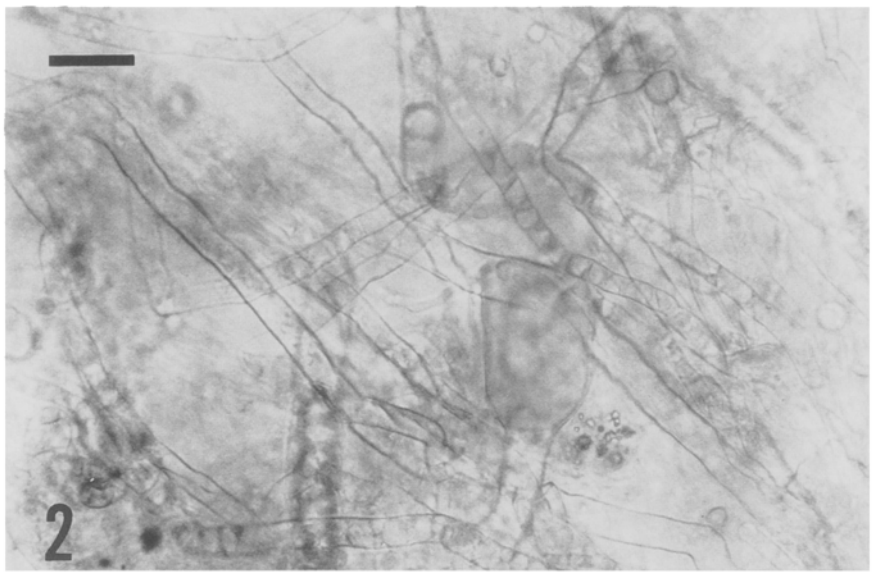
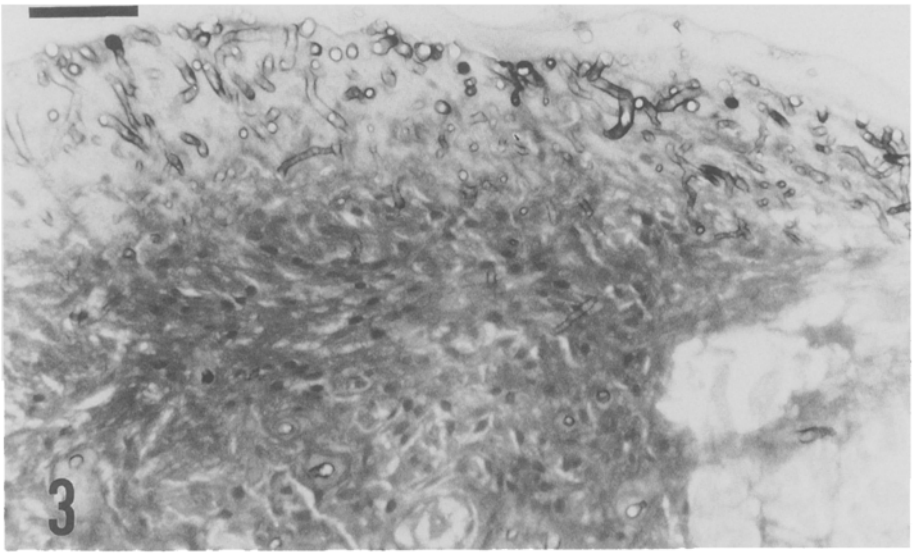


Fig. 1. Gross appearance of the diseased coonstripe shrimp, *Pandalus hypsinotus.* Note the muscle with whitish color (arrow). Scale: 42 mm.
Fig. 2. Hyphae observed in the whitened muscle of the infected shrimp. Scale: 20 μm.

processed for paraffin sections. Sections of 3-5 μm thick were stained with hematoxylin and eosin (H & E), using routine histological methods. Grocott's method was also used.

Identification The present strain was incubated in PYGS broth at 15°C for three days for morphological observation. To observe zoospore discharge, mycelia were washed three times with sterilized artificial sea water (Aqua-Ocean®, Japan Pet Drugs Co.), then placed in a Petri dish containing sterilized artificial sea water and incubated at 15°C and 25°C to induce zoospore formation. To observe the liberation of zoospores, a thallus undergoing zoospore formation was put onto a slide and covered with a coverslip. The shape of the zoospores was observed in a drop of zoospore suspension with a drop of formalin under a microscope. The encysted zoospores were put into PYGS broth and incubated at 25°C to observe germination. The fungus was identified according to Sparrow (1960), Karling (1981) and Hatai and Lawhavinit (1988). Its morphological characteristics and the effect of temperature and NaCl concentration on its growth were compared with those of two strains of *L.*

Fig. 3. Many hyphae observed in the whitened muscle. Grocott stain. Scale: 50 μm.
Fig. 4. Many hyphae observed in the blackened area. Grocott-H & E stains. Scale: 100 μm.
Fig. 5. Hyphae observed in the blood vessels. Grocott stain. Scale: 20 μm.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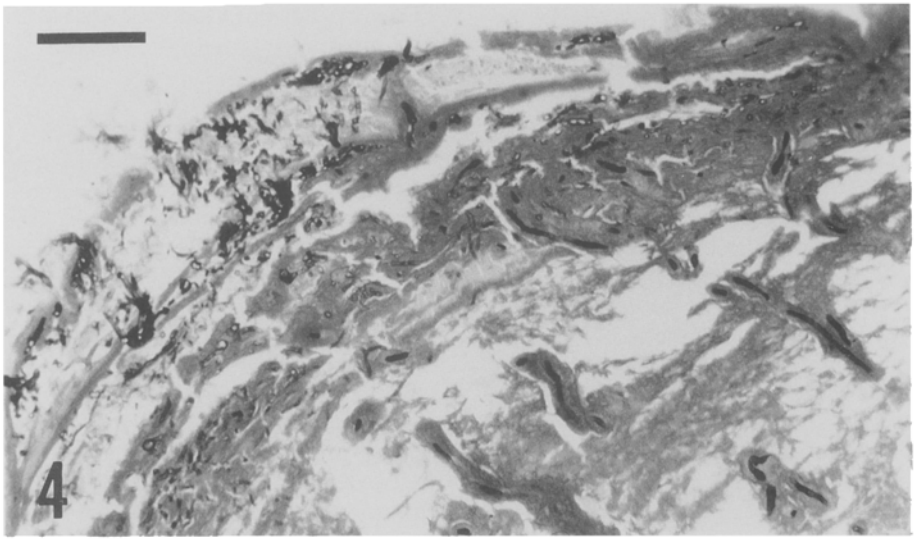
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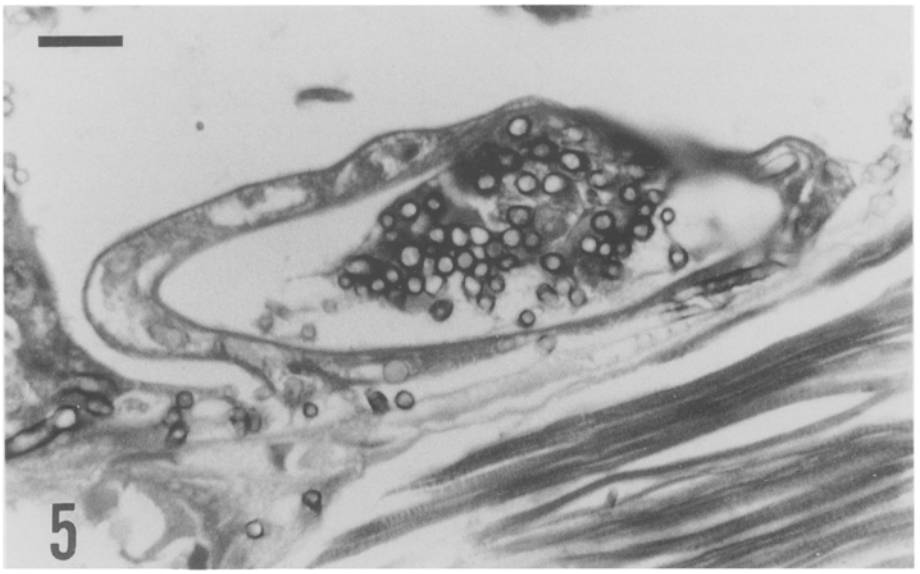
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myophilum, NJM 9131 and NJM 8601.

Effect of temperature on growth All strains were inoculated on PYGS agar and incubated at 15°C for seven days to make a giant colony. PYGS agar discs (5.5 mm diam) cut with No. 2 cork borer from an actively growing edge of the colony were placed in the center of plastic Petri dishes (8.25 cm diam) containing 25 ml of PYGS agar. Six different temperature (5, 10, 15, 20, 25 and 30°C) were used to test the mycelial growth on PYGS agar. The growth rate was determined dialy for seven days after inoculation by measuring the colony diameter at two points with vernier callipers.

Effect of NaCl concentration on growth PYG agar was prepared like PYGS agar but with distilled water replacing artificial sea water, and with nine different concentrations of NaCl (0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 7.0 and 10.0%). Twenty-five ml of each medium was poured into a separate Petri dish and incubated at 15°C. PYGS agar was also prepared as a control medium. Inoculation and measurement of colony diameter were conducted as described above.

Results

Histopathology Many hyphae, which grow along connective tissues among muscle fibers, were observed in the whitened muscle (Fig. 3). Hemocytes infiltrated around hyphae, and some hyphae were surrounded by hemocytes. Melanin pigments deposited on the most exterior layer of the carapace were observed as blackened areas. Hemocytes necrosed with coagulation in the lower layer, which showed an eosinophilic state. An infiltration layer of hemocytes without necrosis was observed deeper in the carapace, diffusing melanized nodules. Numerous hyphae were also present in the blackened lesions (Fig. 4). Hypahe growing along muscle fibers had invaded blood vessels (Fig. 5), producing hyphal clusters. Hyphal growth was not observed in internal organs like the mid-gud gland.

Identification Vegetative hyphae growing in PYGS broth were nonseptate, with numerous oil grobules, branched and 3-8 μm diam. The diameter of the hyphae in the artificial medium was somewhat more uniform than those in the tissue of the shrimps, and did not vary at several incubation temperatures. Zoospore formation occurred within 12-24 h after a thallus was transferred into sterilized artificial sea water. In the process of zoospore formation, protoplasma with numerous oil grobules in the thallus moved into the gelatinous vesicle formed at the orifice of the discharge tube. Mass protoplasma in the vesicle was divided into individual zoospores with two flagella. Vesicles were produced at the top or lateral side of the hyphae. Discharge tubes were 33-242 μm long, 3-4 μm diam, and vesicles were spherical, 25.5-51 μm diam. Zoospores moved in the vesicle slowly before liberation. Release of zoospores occurred when the vesicle was broken by active zoospore movement. Zoospores were laterally biflagellate, pyriform to subglobose, 5-7.7 (av. 6.7) × 7.7-12.8 (av. 10.3) μm, and monoplanetic. Some zoospores were not divided individually, but liberated from the vesicle and encysted as one spore. Zoospores encysted after several minutes' to several hours' swimming. Encysted zoospores were spherical, 5-9.7 (av. 7.7) μm diam. Germination was observed within 2 h after zoospores were encysted (Fig. 6). The fungus was holocarpic and endobiotic. No sexual reproduction was observed. A thallus cultured in PYGS broth for long periods could not produce zoospores after it was transferred into sterilized sea water.

Effect of temperature on growth Growth rate of the present strain was similar to those of strains NJM 8601 and NJM 9131. Optimum growth temperature of the three strains, NJM 9331, NJM 8601 and NJM 9131, was 25-30°C, but they also showed slight growth at 5 and 10°C.

Effect of NaCl concentration on growth There was no difference in growth on PYG agar with various concentrations of NaCl between the present strain and the two strains of *L. myophilum* isolated previously. Optimum NaCl concentration for these strains was 0.5-1.0%. They did not grow on PYG agar with 7-10% NaCl, but did grow slightly with 4-5% NaCl.

Discussion

The fungus isolated from juvenile coonstripe shrimps had the following morphological characteristics: the thallus was holocarpic and endobiotic, and zoospores were produced in the vesicle at the orifice of discharge tube. The fungus was identified as Oomycetes, Lagenidiales, genus *Lagenidium.* The present strain, NJM 9331, was placed in *L. myophilum* acccording to the following characteristics: the method of releacing zoospores, the size of zoospores (av. 6.7 × 10.3 μm) and encysted zoospores (av. 7.7 μm), and the size of vesicles and discharge tubes; these were closely similar to those of *L. myophilum* reported previously. Optimum growth temperature and NaCl concentration of the present strain were 25-30°C and 0.5-1%, respectively, similar to those of the two previously isolated strains of *L. myophilum*, NJM 9131 and NJM 8601. It is known that *L. myophilum* is pathogenic toward adult northern shrimps (Hatai and Lawhavinit, 1988), adult Hokkai shrimps, *Pandalus kessleri* Czerniavski (Hatai, unpublished), and larval coonstripe shrimps. *L. myophilum* infections have only been reported in Japan, and these shrimps of the genus *Pandalus* are known to live only in the deep areas of the sea near to Japan. This is the first finding of fungal infection due to *L. myophilum* in juvenile coonstripe shrimp. As mentioned above, these hosts seemed to be highly sensitive to *L. myophilum.*

L. callinectes, however, known as a parasite of crustaceans, has been reported in the following marine organisms: eggs of the blue crab (Couch, 1942; Rogers-Talbert, 1948; Bland and Amerson, 1973), eggs of the barnacle (Johnson and Bonner, 1960), and the surface of marine algae (Fuller et al, 1964). Histopathologically, numerous nonseptate hyphae grew in the whitened muscle and the blackened areas of the surface of the body, and these hyphae were observed to have similar charac-

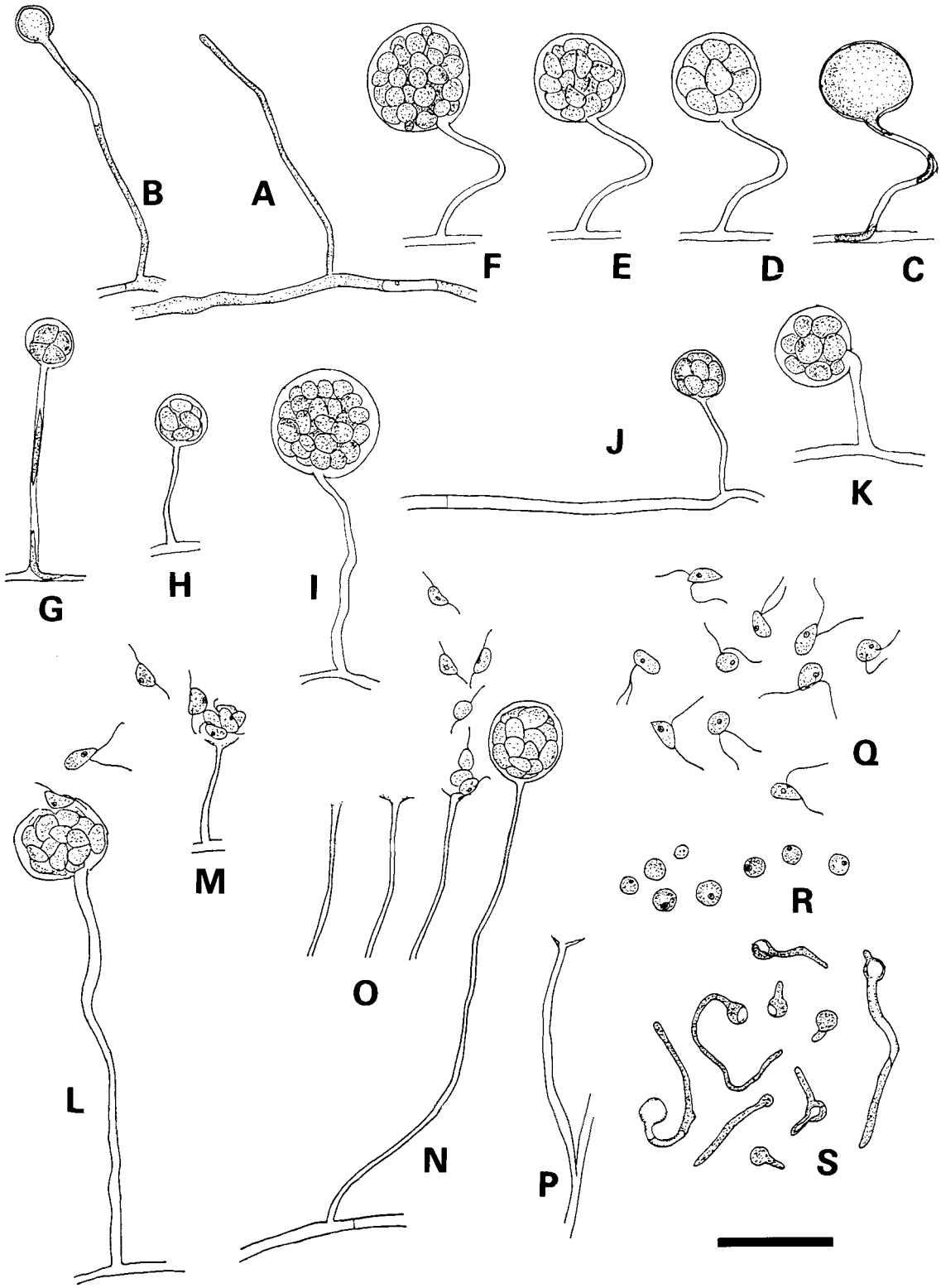

Fig. 6. Zoospore formation of *L. myophilum* NJM 9331 isolated from juvenile coonstripe shrimp. A. Discharge tube formation from hypha; B. Vesicle formation; C-F. Zoospore formation in a vesicle; G. Vesicle formation; H-K. Matured vesicles; L-P. Releace of zoospores; Q. Swimming zoospores, laterally biflagellate; R. Encysted zoospores; S. Germination. Scale: 50 μm.

teristics to each other. From this observation, fungal growth was thought to occur at the blackened lesions first and progress into the muscle. Some hyphae surrounded by numerous melanized hemocytes were observed in the blackened area. This histopathological response appeared to be similar to that reported in adult northern shrimps. The present strain could grow in PYG broth with 0–5% NaCl, and thus is not exclusively a marine fungus.

Adult northern shrimps infected by *L. myophilum* showed low mortality. However, the mortality of the larvae of coonstripe shrimps was approximately 100%, and that of juvenile coonstripe shrimps was approximately 70%. The difference in mortality rates seems to depend on the size and defence reaction of the shrimps. Shrimps surviving a fungal infection might become carriers of the fungus, and act as a source of fungal infections in eggs or larvae with low defence reactions.

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