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Effects of pH and temperature on growth of *Saprolegnia diclina* and *S. parasitica* isolated from various sources

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***Saprolegnia diclina* and *S. parasitica* isolated from three sources could germinate in strong acidic conditions. Growth ability correlated with the species of fungi rather than with the sources from which they were isolated. *S. diclina* isolates appeared to germinate at a pH condition as low as 3.5, whereas *S. parasitica* isolates could not germinate at below pH 3.8. *S. parasitica* isolates from visceral mycoses still showed good growth at 30°C, whereas other isolates did not. Also, *S. parasitica* isolates from visceral mycoses produced more abundant motile zoospores, and continued to do so for a longer period of time (28 d), than *S. parasitica* isolates from external saprolegniasis and *S. diclina* isolates.**

Key Words——pH; *Saprolegnia diclina*; *Saprolegnia parasitica*; temperature.

There have been many reports on fungal infections caused by *Saprolegnia* among the fish population. *S. diclina* Humphrey and *S. parasitica* Coker are the two dominant species which have been detected from diseased fish. These species have been isolated from the external lesions of saprolegniasis-infected fish. *S. parasitica*, in particular, appears to be largely responsible in such cases (Willoughby, 1978; Willoughby and Roberts, 1992). *S. diclina* has been widely obtained from various sources. In visceral mycotic infections, *S. diclina* has been obtained markedly more often than with *S. parasitica*. *S. diclina* has been isolated from the visceral organs of various species of fish, including amago salmon, *Oncorhynchus rhodurus* Jordan et McGregor, and Atlantic salmon fry, *Salmo salar* L. (Hatai and Egusa, 1977; Bruno and Stamps, 1987). Also, specimens of *S. diclina* from the gastro-intestinal tract of rainbow trout, *O. mykiss* (Walbaum), and ayu, *Plecoglossus altivelis* Temminck et Schlegel, have been isolated and maintained in our laboratory. Hitherto, only one report has been published concerning the isolation of *S. parasitica* from visceral organs (Wada et al., 1993).

The present study investigated the effects of pH and temperature on the physiological properties of the two species that may be related to parasitic activity. Specimens were isolated from various sources: external saprolegniasis, visceral mycoses, and fishpond water. Unexpected data discovered in the course of the study which may be useful as taxonomic criteria supporting segregation of the two species are also described.

Materials and Methods

Fungal isolates *Sapralognia diclina* and *S. parasitica* isolates used in all experiments were collected from fish farms or fishery stations in Japan. Details of the isolates are shown in Table 1. Of the 17 isolates, 1 isolate of *S. diclina* and 7 isolates of *S. parasitica* were obtained from lesions on the body surface of saprolegniasis-infected fish, 4 isolates of *S. diclina* and 3 isolates of *S. parasitica* from tissues of the gastro-intestinal tracts of visceral mycotic-infected fish, and 2 isolates of *S. diclina* from fishpond water. The fungi were maintained on GY agar according to Kitancharoen et al. (1995), and subcultured at month intervals. A single-spore culture of each isolate was obtained by diluting zoospore suspensions to adequately low zoospore numbers, spreading them onto 90 × 20 mm Petri dishes containing 20 ml of GY agar, then incubating them at 20°C. After 16–20 h, a single young germinating thallus of each isolate was transferred to fresh agar medium.

pH and zoospore germination The zoospore suspension of each isolate was obtained by inoculating the advancing edge of the mycelia on an agar block (about 5 × 5 mm in size) into a small plastic Petri dish (50 × 15 mm) containing 10 ml of GY broth, which was then maintained at 20°C for 24 h. The mycelia were washed in successive baths of sterilized tap water, then transferred to sterilized tap water and kept at 20°C for 24 h. The numbers of zoospores in the suspensions of all isolates were counted and adjusted to 1×10^4 spores/ml. A 100 μl portion of zoospore suspension of each fungal isolate was inoculated into Petri dishes containing 10 ml of GY broth at various pHs, as shown in Table 2. The media were adjusted

Table 1. Isolates of *S. diclina* and *S. parasitica* used in this study.

Isolate	Source of isolate[a)]	Site[b)]	Location	Yr isolated
S. diclina				
NJM 0005	coho salmon	E	Miyagi	1986
NJM 1010	yamame salmon	I	Tokyo	1986
NJM 1014	yamame salmon	I	Tokyo	1986
NJM 8536	culture pond water	—	Kanagawa	1985
NJM 8540	culture pond water	—	Gifu	1985
NJM 9105	ayu	I	Tochigi	1991
NJM 9222	ayu	I	Tochigi	1991
S. parasitica				
NJM 8407	rainbow trout	E	Kanagawa	1984
NJM 8604	yamame salmon	E	Miyagi	1986
NJM 8737	rainbow trout	E	Gunma	1987
NJM 9101	ayu	I	Tochigi	1991
NJM 9102	ayu	I	Tochigi	1991
NJM 9106	ayu	E	Wakayama	1991
NJM 9225	ayu	I	Tochigi	1992
NJM 9302	pejerrey	E	Tochigi	1993
NJM 9305	ayu	E	Tochigi	1993
NJM 9522	pejerrey	E	Tochigi	1995

a) Scientific names of fish: coho salmon, *Oncorhynchus kisutch*; yamame salmon, *Oncorhynchus masou*; ayu, *Plecoglossus altivelis*; rainbow trout, *Oncorhynchus mykiss*; pejerrey, *Odonthetes bonariensis*.
b) E: Isolated from lesions on body surfaces of saprolegniasis-infected fish. I: Isolated from visceral organs of internal mycotic-infected fish.

to the required pH levels after autoclaving, then filtered with 0.45 μm Millipore filter paper (Whatman). The inoculated plates were incubated at 20°C for 7 d to observe germination and growth. pH changes were qualitatively measured with Toyo test paper (Toyo Roshi) after the experiment.

Temperature and vegetative growth The following test was conducted on each isolate. The advancing edges of colonies cultured at 20°C for 2 d on 20 ml of GY agar were cut with a no. 2 cork borer (5.5 mm in diam) and placed onto the center of 90 × 20 mm Petri dishes containing 20 ml of GY agar, then incubated at 10, 20 and 30°C. The radial growth of the colonies was measured and the mean of four perpendicular radii was calculated.

Temperature and asexual reproduction Mycelia of the fungal isolates cut from GY agar blocks were grown in GY broth at 20°C for 24 h. The mycelia were washed with sterilized tap water as described above, then replaced in approximately the same amount into small Petri dishes containing 10 ml of sterilized tap water and held at 10, 20 and 30°C. The number of motile zoospores was determined with a Neubauer counting chamber (Erma^R) every day for 1 wk, following which the occurrence of motile zoospores was checked weekly for 1 mo.

Results

pH and zoospore germination The effect of pH on growth of *S. diclina* and *S. parasitica* isolates was determined in terms of germinating and growth ability. As shown in Table 2, all isolates of both species were capable of germinating and growing in acidic conditions. All isolates tested grew rapidly at pH 4.8, covering the surface of the Petri dishes 3–4 d after inoculation. At pH 3.8, *S. diclina* and *S. parasitica* exhibited obvious differences in growth performance. All isolates of *S. parasitica* both from external lesions and visceral organs formed small dense colonies with contorted, irregularly short branched hyphae (Fig. 1A), whereas all isolates of *S. diclina* displayed normal morphology (Fig. 1B). All isolates of *S. diclina* were also able to germinate and grow at lower pH (pH 3.5), but *S. parasitica* were not. With the growth of mycelia, the pH of the media gradually increased.

Temperature and vegetative growth All *Saprolegnia* isolates showed similar growth at temperatures of 10 and 20°C. However, at 30°C the isolates could be clearly segregated into two groups, as illustrated in Fig. 2. Group 1, composed of 6 isolates from visceral mycotic infected fish (NJM 1010, NJM 9101, NJM 9102, NJM 9105, NJM 9222 and NJM 9225) and 1 isolate (NJM 8536) from fishpond water, showed rapid growth. Group 2, composed of 8 isolates from external saprolegniasis-infected fish (NJM 0005, NJM 8407, NJM 8604, NJM 8737, NJM 9106, NJM 9302, NJM 9305 and NJM 9522), NJM 8540 from fishpond water and NJM 1014 from visceral mycotic infected fish, showed little or no

Table 2. Germination and growth of *S. diclina* and *S. parasitica* isolates at various pH levels in GY broth after incubation at 20°C for 2 d.

Isolate	pH		
	3.5	3.8	4.8
S. diclina			
NJM 0005	+[a)]	++	+++
NJM 1010	+[a)]	++	+++
NJM 1014	+[a)]	++	+++
NJM 8536	+[a)]	++	+++
NJM 8540	+[a)]	++	+++
NJM 9105	+[a)]	++	+++
NJM 9222	+[a)]	++	+++
S. parasitica			
NJM 8407	−	+[a)]	+++
NJM 8604	−	+[a)]	+++
NJM 8737	−	+[a)]	+++
NJM 9101	−	+[a)]	+++
NJM 9102	−	+[a)]	+++
NJM 9106	−	+[a)]	+++
NJM 9225	−	+[a)]	+++
NJM 9302	−	+[a)]	+++
NJM 9305	−	+[a)]	+++
NJM 9522	−	+[a)]	+++

+, ++, +++: Degrees of mycelial growth.
a) Hyphae displayed abnormal growth features.
−: No germination revealed or failure of development of germ tubes into hyphae.

growth. The 10 isolates of group 2, which failed to grow or grew slowly, were placed onto fresh GY agar at 20°C after 7 d, at which point they began to show normal growth.

Temperature and asexual reproduction Most isolates of *Saprolegnia* produced abundant zoospores at 10°C, except for NJM 8536 which produced no motile zoospores: only encysted spores and germinating spores were detected during the observation period (Fig. 3). Motile zoospores of 9 isolates were numerous until about 7 d. The occurrence of motile zoospores of all *S. parasitica* isolates continued for about 1 mo, but all *S. diclina* isolates stopped producing zoospores after 7 d. At 20°C, production of motile zoospores of all isolates diminished compared with that at 10°C. Also, the number of motile zoospores decreased more rapidly, and the occurrence of motile zoospores of all isolates stopped after 7 d (Fig. 4). The results at 30°C (Fig. 5) obviously differed from those at the 2 lower temperatures, with the number of motile zoospores and period of zoospore production markedly reduced. All isolates ceased zoospore production after 5 d.

Discussion

The most suitable pH for *Saprolegnia* tended to be acidic conditions of pH 5.0–5.5 for mycelial growth and pH 6.2 for zoospore production (Peduzzi et al., 1991). *S. ferax* (Gruith.) Thuret. isolated from the gut of blackfly larvae, *Simulium vittatum* Zetterstedt, grew best in the temperature range of 15–26°C with an optimal pH of 5.6 (Nolan, 1976). Also, the optimal temperature and pH range of *S. megasperma* Coker isolated from nematode, *Neomesomeris flumenalis*, were 13–19°C and 5.2–5.7, respectively (Nolan, 1975). The present study reveals that zoospores of the two species of *Saprolegnia* studied are capable of withstanding and growing in strongly acidic conditions. This suggests that if zoospores pass through the gastro-intestinal tract, they are able to germinate, and hyphae can penetrate the tissue and invade the contiguous organs without their viability being affected by the acidity in the stomach of the fish. Also, the pH tolerance of the two species clearly differed according to the species rather than the source of the isolates. Isolate NJM 9302, identified as *S. diclina* Type 1 by Kitancharoen et al. (1995), seemed more similar to the group of *S. parasitica* than to the group of *S. diclina*.

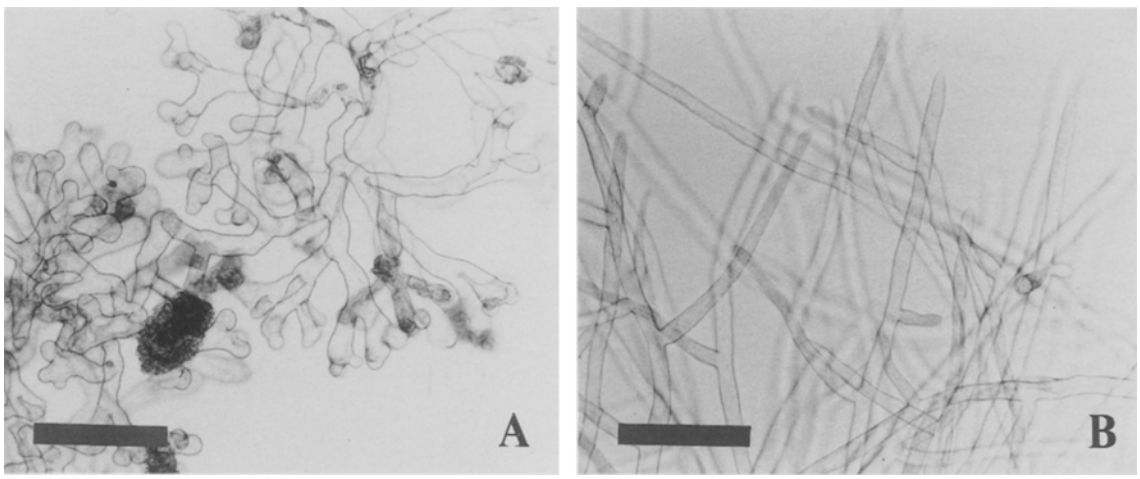

Fig. 1. Mycelia of *Saprolegnia* growing in GY broth, pH 3.8 at 20°C for 4 d.
A. Mycelia of *S. parasitica*; B. Mycelia of *S. diclina* (Scale bars = 200 μm).

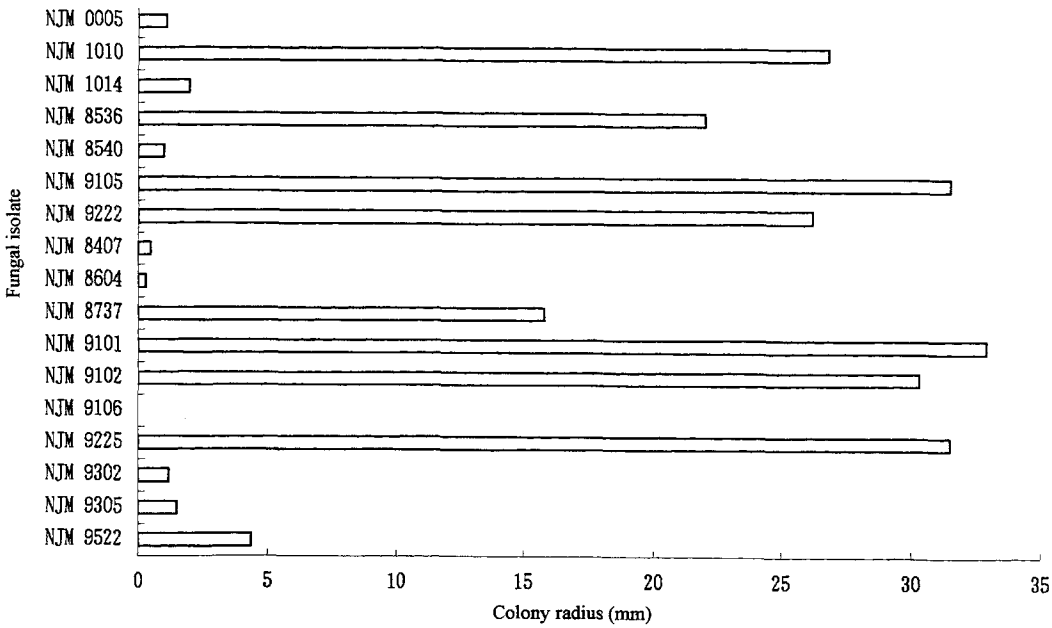

Fig. 2. Colony radial growth of *S. diclina* and *S. parasitica* isolates at 30°C on GY agar. Note the isolates were segregated into two groups with markedly different growth.

Moreover, the results of pH study made it clear that isolate NJM 9302 was more similar to the *S. parasitica* group than to the *S. diclina* group. Other isolates of *S. diclina* Type 1 also exhibited identical properties (unpublished data).

An unexpected result of the study was the discovery that the data also appear to have some taxonomic value. In addition to the classification of the two species based on such characteristics as the hairs on the secondary spore cysts and germination (Pickering et al., 1979; Beakes, 1983; Hatai et al., 1990), or the form of sexual reproduction, this study offered an outstanding possibility for a new method for identification and classification of *S. diclina* and *S. parasitica*. pH levels of 3.5 and 3.8 showed strikingly different effects on the growth of the two species. *S. diclina* was capable of growing in stronger acidic conditions than *S. parasitica*. This also suggested the reason why *S. diclina* was found to be associated with visceral mycoses more frequently than *S. parasitica*. The abnormal characteristic of hyphae which occurred in low pH conditions is suggestive of induction of cytoplasmic protein synthesis when the environment is not suitable. Turian et al. (1991) stated that the germ tubes from germinated zoospores of *S. parasitica* were acidified. Accordingly, the pH of GY medium gradually increased during the experiment, and clearly increased after the fungal colonies were developed. The release of the end products, alkaline substances such as ammonia or urea, when the fungi assimilate nitrogen sources in the medium through the metabolic pathways may explain this phenomenon.

Saprolegniasis tends to occur seasonally (Olah and Farkas, 1978), usually affecting fish at low temperature most severely, which is ascribed to the increased stress in fish, and creation of favorable conditions for the

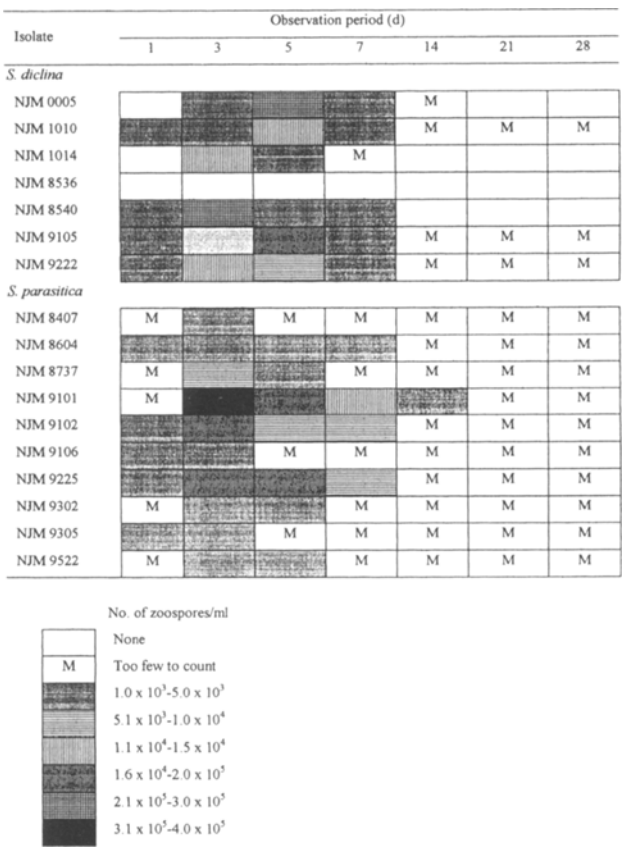

Fig. 3. Production of zoospores in sterilized tap water at 10°C.

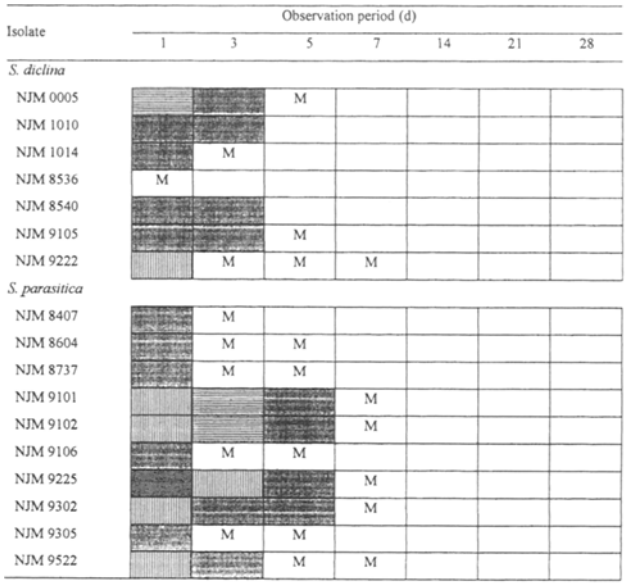

Fig. 4. Production of zoospores in sterilized tap water at 20°C.

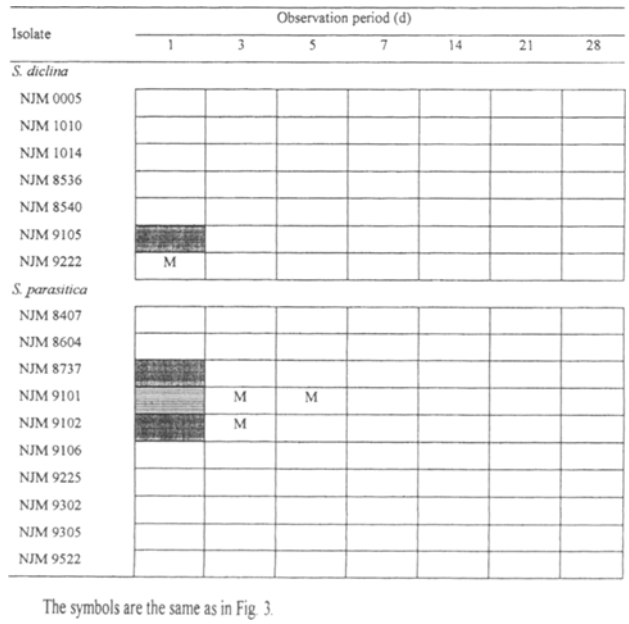

Fig. 5. Production of zoospores in sterilized tap water at 30°C.

proliferation of *Saprolegnia* (Bly et al., 1993). Schaefer et al. (1981) reported that mortality of rainbow smelt caused by *Saprolegnia* sp. occurred at a temperature of 15°C. Accordingly, an artificial infection test on moonfish of the *Saprolegnia* isolates used in this study did not succeed at a temperature of 20°C (Yuasa, 1995). In this study, in the temperature range of 10–20°C, most isolates produced sporangia and motile zoospores, as expected, but isolate NJM 8536 was an exception. This isolate dominantly produced encysted spores and germinating spores, suggesting that the motile stage, which plays an important role in parasitizing, was reduced. A pathogenicity test against rainbow trout by Yuasa and Hatai (1995) revealed that isolate NJM 8536 is saprophytic. At 10°C, motile zoospores of most isolates continuously appeared for about 1 mo, while at 20°C they disappeared after 7 d. All isolates of *S. parasitica* and *S. diclina* from visceral mycoses produced numerous motile zoospores. At 30°C, the production of motile zoospores was clearly inhibited in most isolates. Similarly, Dieguez-Uribeondo (1995) reported that *S. parasitica* from brown trout produced no zoospores at 30°C. This indicates that the abundance and duration of motile zoospore production are inversely related with temperature. The normal life cycle of *Saprolegnia* is disturbed by high temperatures, since the number of motile zoospores, which are crucial for parasitizing, and the period of zoospore production decreased. Most isolates from visceral mycoses showed a broader spectrum of thermo-tolerance in vegetative growth than isolates from external saprolegniasis and from fishpond water. At 30°C, all isolates of the two species from visceral mycoses exhibited rapid growth of vegetative hyphae, whereas the isolates from external saprolegniasis did not. Isolate NJM 8536, a saprophytic isolate from fishpond water, showed rapid growth at this temperature, differing from NJM 8540, another saprophytic isolate also from fishpond water. In zoospore production, the two isolates also appeared to differ in the occurrence of motile zoospores.

This study leads to the conclusion that the characteristic of pH-tolerance of the fungi is correlated with fungal species, whereas the temperature-tolerance of the fungi is related to the source from which they were isolated.

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