Haliphthoros spp. from spawned eggs of captive mud crab, *Scylla serrata*, broodstocks

Eduardo M. Leaño

Aquaculture Department, Southeast Asian Fisheries Development Center, Tigbauan, 5021 Iloilo, Philippines; e-mail: edleano@yahoo.com

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Monitoring of the fungal flora of spawned eggs of captive mud crab, *Scylla serrata*, was conducted in several hatchery runs at the Aquaculture Department of Southeast Asian Fisheries Development Center in Iloilo, Philippines. Quantification of the egg mycoflora revealed the dominance of oomycetes, particularly *Haliphthoros* spp. among spawners which aborted their eggs prior to hatching. Two species of *Haliphthoros* (*H. philippinensis* and *H. milfordensis*) were identified from the 24 isolates collected. *Haliphthoros milfordensis* was the dominant species. Physiological studies on vegetative growth and sporulation of the two species show that *H. philippinensis* have wider optimal range for salinity and temperature requirements than *H. milfordensis*, especially in sporulation. The pathogenicity study showed that only *H. milfordensis* was pathogenic to spawned eggs of *S. serrata*, while *H. philippinensis* was not. Infection of *S. serrata* eggs by *H. milfordensis* was observed starting at two days after inoculation of zoospores with 2-5% infection rate, reaching up to 10% at five days post-inoculation.

Key words: crab eggs, *Haliphthoros*, oomycetes, sporulation, vegetative growth.

Introduction

The Mud Crab, *Scylla serrata*, also known as Mangrove Crab, is an important aquaculture commodity in the Indo-Pacific region, where commercial production currently relies on wild-caught seeds for stocking (Keenan, 1999). Recent advances on hatchery operations had been undertaken to cope with the increasing demand for crablets for grow-out culture (Mann *et al*., 1999; Quinitio *et al*., 1999). Mass production of juveniles is, however, still hampered by the low and inconsistent larval survival in the hatchery. At the Aquaculture Department of Southeast Asian Fisheries Development Center (SEAFDEC AQD) in Iloilo, Philippines, hatchery operations of Mud Crab started in 1996 with recorded survival of 1-10% from hatchlings to crablet stage (Triño *et al*., 1999). One of the major problems encountered, aside from low supply of berried females (Latiff and Musa, 1995), are microbial infections (e.g. bacterial and fungal) (Bian and Egusa, 1980; Nakamura *et al*., 1995,

Lavilla-Pitogo *et al.*, 2001; Quinitio *et al.*, 2001). Thus, monitoring of the microflora (bacteria and fungi) of spawned eggs and larvae of the mud crab was undertaken from July 1999-February 2000.

Quantification and characterization of the mycoflora of spawned eggs revealed the dominance of pathogenic oomycetes (*Haliphthoros* and *Lagenidium*) in some spawners. This condition often leads to abortion or resorption of the egg mass prior to hatching. This study was therefore conducted in order to: 1) characterize the *Haliphthoros* species isolated from the egg mass of Mud Crab spawners, with regard to their growth and sporulation; and, 2) assess the pathogenicity of selected isolates to spawned eggs of *S. serrata*.

Materials and Methods

Isolation and identification

Eggs from berried *Scylla serrata* were aseptically collected (every two days from the day of spawning) and placed in sterile plastic bottles (Nalgene). In the laboratory, the eggs were weighed and homogenized (0.1 g eggs ml^{-1}) in sterile natural seawater (NSW) supplemented with antibiotics (1000 U ml^{-1} Penicillin G, and 500 µg ml^{-1} streptomycin sulphate). Homogenized eggs were allowed to stand for 30 minutes prior to inoculation (by pour plate method) onto peptone yeast-extract glucose seawater (PYGS) agar (composed of 1.25 g mycological peptone (BBL), 1.25 g yeast-extract (BBL), 3 g glucose (Ajax), 12 g agar (BBL), 1 l NSW) supplemented with antibiotics. Two dilutions (10^0 and 10^1) were used per egg sample.

Fungal colonies were counted after 48 hours incubation at room temperature (28-30 C). Dominant colonies were isolated and transferred onto fresh PYGS agar. Oomycetes were further sub-cultured into PYGS broth and incubated at room temperature for 24 hours. The resulting mycelial mats were then washed in sterile NSW and transferred into fresh NSW to induce sporulation. Sporulation was monitored daily until day 5. *Haliphthoros* isolates were identified based on the descriptions of Vishniac (1958), Hatai *et al.* (1980), and Nakamura and Hatai (1995).

Physiological study

Two representative isolates were used namely: *Haliphthoros milfordensis* Vishniac (isolate S30-2) and *H. philippinensis* Hatai, Bian, Batic. & Egusa (isolate T18-1).

Vegetative growth. The isolates were sub-cultured on PYGS agar. After 5 days of incubation (25 C), agar discs (5 mm diam.) were cut from the colony

margin. PYGS broth was prepared at different salinity levels using distilled water (0‰) and artificial seawater (ASW) using sea salts (Sigma) for salinities from 10-40‰ (at 5‰ intervals). One disc was inoculated into each 50 ml PYGS broth (in 125 ml conical flasks). Inoculated flasks (in triplicate) were then incubated at different temperature levels (15, 20, 25, 30 and 35 C) for 5 days and total biomass (mg dry weight) determined.

Sporulation. The isolates were sub-cultured on PYGS broth and incubated at 25 C for 24 hours. Mycelial mats were then harvested and washed in sterile ASW prior to inoculation into sterile distilled water (0‰) and ASW (10-40‰) in Petri-dishes. Triplicate plates were incubated at different temperature levels (15, 20, 25, 30 and 35 C) for up to 5 days. Sporulation was monitored daily.

Pathogenicity study

Mass production of zoospores. Zoospores of the two isolates (S30-2 and T18-1) were mass produced using the optimum salinity and temperature requirements for sporulation. A zoospore density of approximately 10^4 zoospores ml^{-1} was used for the infection experiment.

Infection experiment. Eggs from berried *Scylla serrata* were aseptically collected and examined microscopically to assess the health status of the eggs. Eggs classified as "normal" were washed in sterile NSW, and inoculated into 250 ml conical flasks containing 200 ml sterile NSW at a density of 1 mg eggs ml^{-1}. The zoospore suspension was then added into the flask at a dose of 200 zoospores ml^{-1}. Control flasks were inoculated with sterile NSW only. Mild aeration was provided throughout the experiment (5 days) at ambient temperature (27-29 C). Representative samples from each flask were monitored daily for the presence of infected eggs, and percent (%) infected eggs was estimated. Two experimental runs were conducted.

Reisolation. Infected eggs were washed in sterile NSW and inoculated into PYGS broth with antibiotics. After incubation (24-48 hours), the isolates were transferred onto PYGS agar until axenic cultures are obtained. Species identification was reconfirmed through induced sporulation.

Statistical analysis. Data on vegetative growth were analysed using two-way analysis of variance, and significant differences among treatment means were compared using the Duncan's multiple range test (Zar, 1996).

Table 1. Fungal load (CFU* $0.1g^{-1}$) of spawned eggs of *Scylla serrata* cultured in captivity.

Sample	Day after spawning					Remarks
	0	2	4	6	8	
S33	5×10^0	5.2×10^1	9×10^1	8.5×10^1	7×10^1	Treated with 0.1 ppm Treflan at day 2; successfully hatched at day 10
S31			1.5×10^1	3×10^1	3.5×10^1	Successfully hatched at day 10
T18	1.1×10^2	4.8×10^2	6.4×10^3	4.6×10^3	1.6×10^3	Pure colonies of *Haliphthoros* from day 4; eggs aborted at day 10
S30	8.3×10^1	2.3×10^2	3.5×10^3	3.3×10^3	2.2×10^3	Dominant *Haliphthoros* colonies; aborted at day 9
S33**	4×10^0	7×10^2				Pure colonies of *Haliphthoros* at day 2; aborted at day 3.

* CFU = colony forming units; **second spawning cycle.

Results

Isolation and Identification

Table 1 shows the fungal load of *Scylla serrata* eggs spawned in captivity. Three of the five spawners sampled aborted their eggs due to the dominance of *Haliphthoros* spp. Higher fungal loads were also observed on these spawners (10^2-10^3 colony forming units [CFU] per 0.1 g eggs) compared to the other two spawners (10^1 CFU per 0.1 g eggs) where the eggs successfully hatched at 10 days post spawning. A total of 24 isolates of *Haliphthoros* were obtained from spawners and maintained in axenic cultures.

All *Haliphthoros* isolates have similar vegetative structures. Colonies on PYGS agar appeared whitish and filamentous, attaining a diam. of 40-60 mm after 6 days incubation at room temperature. Microscopically, hyphae were hyaline, stout, non-septate, and irregularly branched, 18-35 µm in diam., sometimes reaching up to 50 µm in older cultures. In broth culture and during induced sporulation, two groups were distinguishable as follows: (1) Thalli grew at the surface of the broth medium forming a firm mat with age; hyphal fragmentation, zoospore differentiation and zoospore release were observed within 24 hours after transfer to sterile NSW. (2) Thalli grew in the broth medium without forming a mat on the surface; hyphal fragmentation observed after 24-48 hours and zoospore differentiation and release after 48 hours.

Zoospores were released through discharge tubes formed from the zoosporangia. The discharge tubes were either straight, wavy, or coiled, 80-530 µm in length and 6-8 µm in diam. Release of zoospores from an opening on the zoosporangial wall was also observed (rarely) in the first group of isolates.

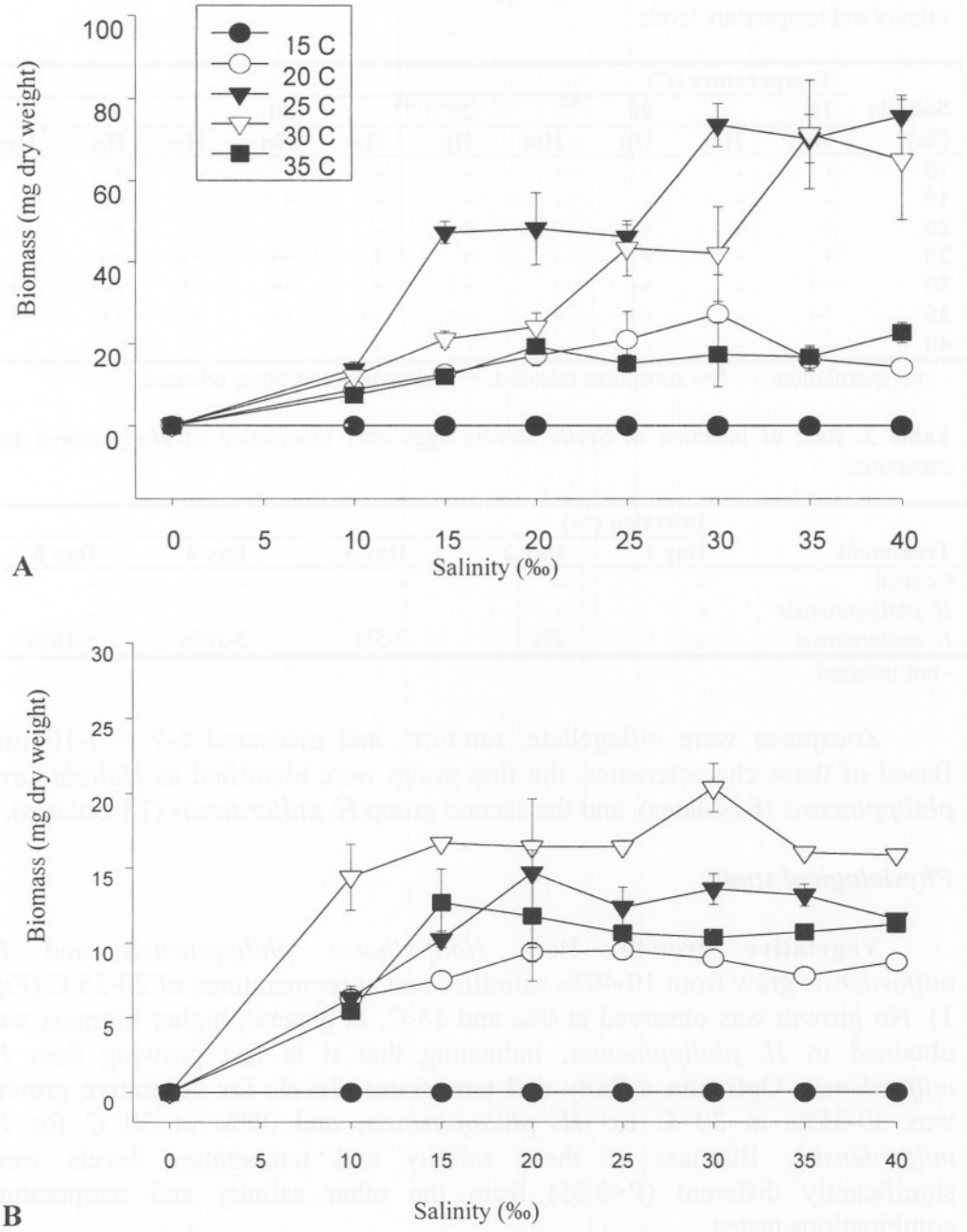


Fig. 1. Vegetative growth (mean ± SEM) of *Haliphthoros philippinensis* (A) and *H. milfordensis* (B) at different salinity and temperature levels after 5 days incubation.

Table 2. Sporulation of *Haliphthoros philippinensis* (Hp) and *H. milfordensis* (Hm) at different salinity and temperature levels.

Salinity (‰)	Temperature (C)									
	15		20		25		30		35	
	Hp	Hm	Hp	Hm	Hp	Hm	Hp	Hm	Hp	Hm
10	-	-	-	-	-	-	-	-	+	-
15	-	-	-	-	-	-	+	+	+	+
20	+	-	+	-	+	-	++	+	++	+
25	+	-	+	+	+	+	++	++	++	+
30	++	-	++	+	++	+	++	++	++	++
35	++	-	++	+	++	++	++	++	++	++
40	-	-	-	-	-	+	-	+	-	+

- : no sporulation; + : few zoospores released; ++ : abundant zoospores released.

Table 3. Rate of infection of *Scylla serrata* eggs after inoculation of *Haliphthoros* spp. zoospores.

Treatment	Infection (%)				
	Day 1	Day 2	Day 3	Day 4	Day 5
Control	-	-	-	-	-
H. philippinensis	-	-	-	-	-
H. milfordensis	-	2%	3-5%	5-10%	5-10%

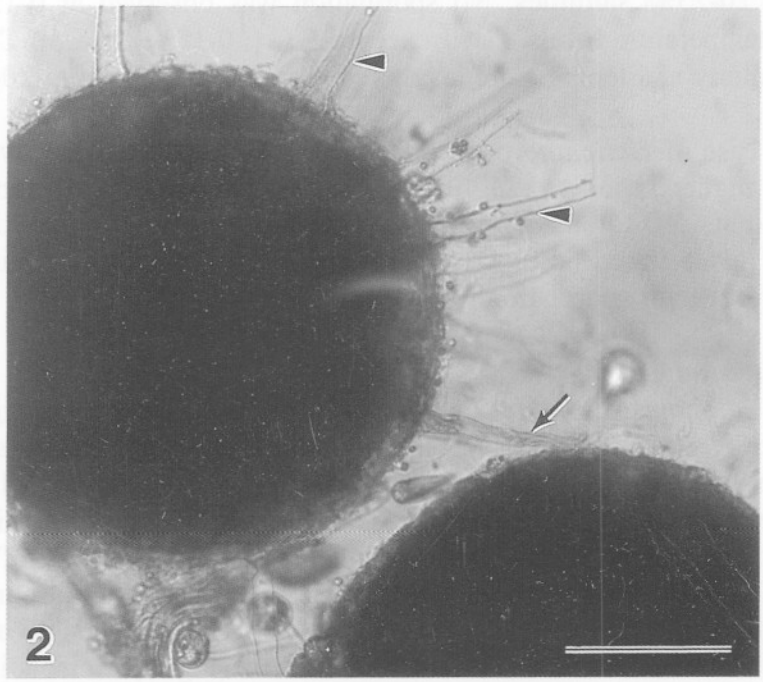
- not infected.

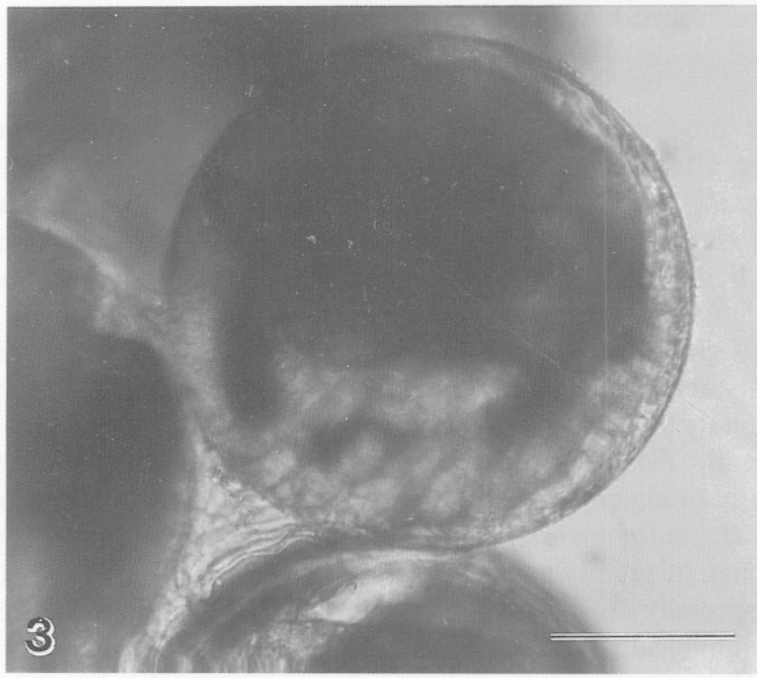
Zoospores were biflagellate, reniform and measured 6-7 × 7-10 µm. Based of these characteristics, the first group were identified as *Haliphthoros philippinensis* (6 isolates), and the second group *H. milfordensis* (18 isolates).

Physiological study

Vegetative growth. Both *Haliphthoros philippinensis* and *H. milfordensis* grew from 10-40‰ salinities and at temperatures of 20-35 C (Fig. 1). No growth was observed at 0‰ and 15 C. In general, higher biomass was obtained in *H. philippinensis*, indicating that it is fast growing than *H. milfordensis*. Optimum salinity and temperature levels for vegetative growth was 30-35‰ at 30 C for *H. philippinensis*, and 30‰ at 30 C for *H. milfordensis*). Biomass at these salinity and temperature levels were significantly different ($P<0.05$) from the other salinity and temperature combinations tested.

Sporulation. *Haliphthoros philippinensis* sporulated over a wide range of salinity and temperature levels compared to *H. milfordensis*. *Haliphthoros philippinensis* produced abundant zoospores at 30-35‰ from 15 to 35 C (Table 2). At higher temperatures (30-35 C), abundant zoospore production was also





Figs. 2-3. *Scylla serrata* eggs. **2.** Infected with *Haliphthoros milfordensis*; note the presence of discharge tubes (arrowheads) releasing the zoospores (arrow). **3.** Uninfected eggs (control treatment). Bars = 100 μm.

observed at 20-25‰ salinities. No sporulation was observed at 0-15‰ and 40‰ at all temperature levels tested except 35 C (10-15‰) and 30 C (15‰). Optimum salinity and temperature levels for sporulation were from 20-35‰ at 30-35 C.

In the case of *Haliphthoros milfordensis*, abundant zoospores were only produced at higher salinities (25-40‰) at 25-30 C (Table 2). No sporulation was observed at 15 C for all salinity levels tested, and at 0-20‰ salinities at all temperature levels tested (except 15-20‰ at 30-35 C). Optimum salinity and temperature levels for sporulation were 30-35‰ at 30-35 C.

Pathogenicity study

Table 3 shows the rate of infection of *Scylla serrata* eggs after inoculation of *Haliphthoros* zoospores. From the two experimental runs, results show that only *H. milfordensis* was pathogenic to *Scylla serrata* eggs. Infection was observed starting at 2-3 days post inoculation with 2-5% infection rate, reaching up to 10% at day 5. Infected eggs show the presence of several discharge tubes extruding from the egg surface to release the zoospores (Fig. 2). Discharge tubes were straight and measured 85-101 μm in length and 8-11 μm in diam. Control treatments remained uninfected (Fig. 3). Reisolation from infected eggs confirmed the presence of *Haliphthoros milfordensis*.

Discussion

In seed production of *Scylla serrata*, mycotic infections caused by oomycetes are often encountered (Bian *et al.*, 1979; Bian and Egusa, 1980; Nakamura *et al.*, 1995). Two species are considered as serious pathogens: *Haliphthoros milfordensis* and *Lagenidium callinectes* Couch (Nakamura and Hatai, 1995). In this study, *Haliphthoros* spp. was found to dominate the fungal population of *Scylla serrata* eggs leading to abortion/resorption of the egg mass. The two species of *Haliphthoros* isolated differ from each other in terms of their salinity and temperature requirements for growth and sporulation, and their pathogenicity to *Scylla serrata* eggs.

In general, *Haliphthoros milfordensis* (the dominant species) have narrower optimal range (salinity and temperature) for growth and sporulation compared to *H. philippinensis*. It was reported that saprotrophic oomycetes have wider range of salinity and temperature tolerance compared to pathogenic oomycetes, especially on sporulation (Leaño, 1999). From the results obtained from the physiological study, it can be deduced that *H. philippinensis* appears to be a saprobic species while *H. milfordensis* is more likely a pathogenic species. Both isolates are obligate marine requiring a minimum salinity of 15‰ for growth and sporulation.

Haliphthoros philippinensis was initially reported to be associated with fungal infection of cultured larvae of the shrimp, *Penaeus monodon*, in the Philippines (Hatai *et al*., 1980). Since then, no study reported the association of this species with any crustacean host. In the study of Hatai *et al*. (1980), the pathogenicity of *Haliphthoros philippinensis* on *Penaeus monodon* larvae was not established, and it was suggested that it is not severely pathogenic as this species is rarely found in shrimp hatcheries. This study is the first report on the association of *Haliphthoros philippinensis* on *Scylla serrata* eggs. The pathogenicity study, however, revealed that it is non-pathogenic. Therefore, they may only occur as saprotrophs on the egg mass of *Scylla serrata* broodstocks.

Haliphthoros milfordensis, on the other hand, had been reported to be associated with mycotic infections of crabs and other marine crustaceans. It is a well-known important fungal pathogen of eggs and larvae of crustaceans (Hatai, 1989). It was initially reported on infected eggs and larvae of oyster drill, *Urosalpinx cinerea* (Vishniac, 1958). Since then, *Haliphthoros milfordensis* was found to be associated with fungal infections of may different hosts: cultured abalone, *Haliotis sieboldii* (Hatai, 1982); postlarvae of lobsters, *Homarus americanus* and *H. gammarus* (Lightner, 1981; Stewart, 1993); ova and larvae of *Scylla serrata* (Kaji *et al*., 1991); and *Penaeus japonicus* with black gill disease (Hatai *et al*., 1992). It was also reported to be capable of infecting eggs of Blue Crab (*Callinectes sapidus*), eggs, embryo and adult stages of Brine Shrimp (*Artemia salina*), adults of Pink Shrimp (*Penaeus duorarum*), and zoea of Marine Crab (*Portunus pelagicus*) (Tharp and Bland, 1977; Nakamura and Hatai, 1995).

The pathogenicity of *Haliphthoros milfordensis* in this study showed that it can infect up to 10% of *Scylla serrata* eggs within five days post inoculation at a concentration of 10^2 zoospores ml^{-1}. Hamasaki and Hatai (1993) also reported that infection in eggs and larvae of *S. serrata* occurred at 10^2-10^3 zoospores ml^{-1} inoculum (using *Haliphthoros*, *Lagenidium* and *Sirolpidium*). Furthermore, infection was evident starting at day 2 post-inoculation, when infected *Scylla serrata* eggs showed the presence of discharge tubes emerging from the egg surface. In *Halocrusticida hamanaensis*, infection of *Scylla serrata* eggs was also observed in the first two days of inoculation (Bian and Egusa, 1980).

The presence of discharge tubes on the infected eggs represents the late stage of infection. Because of the thickness of the egg membranes and their opacity, the intrammatrical hyphae during the early stage of infection could not be observed (Bian and Egusa, 1980). Thus, it can be deduced that infection of *Scylla serrata* eggs occurred earlier than 2 days (probably within 24 hours)

after inoculation of *Haliphthoros* zoospores. Studies on settlement time of zoospores of pathogenic and non-pathogenic oomycetes show that attachment of zoospores on suitable substrata can occur within 5-30 minutes (Gubler and Hardham, 1988; Rand and Munden, 1993; Enkerli *et al.*, 1996; Leaño, 1999). Once attached, germination will take place leading to successful colonization or infection (Leaño, 1999).

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