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Treatment of *Achlya* Infection in Freshwater Seabass, *Lates calcarifer* (Bloch 1790)

LAU LIK MING[1] and KISHIO HATAI[1*]

[1]Microbiology and Fish Disease Laboratory, Borneo Marine Research Institute, Universiti Malaysia Sabah, 88999 Kota Kinabalu, Sabah, Malaysia

Abstract

Achlya infection is a common disease in freshwater-reared fishes. A strain of water mould, *Achlya* sp. IPMB 1403, was isolated from the tail fin of dying freshwater-reared Asian seabass, *Lates calcarifer* (Bloch 1790) fry and was used in the present study for treatment trials. The antifungal effects of seawater and sodium chloride on hyphal growth and zoospore germination were investigated at 25 ºC by using seawater up to 30 ppt and NaCl up to 3.0 %. Seawater at 15 ppt and above and NaCl at 1.5 % and above inhibited hyphal growth, while exposure to seawater at 30 ppt for 2 h or NaCl at 2.0 % for 2 h or 3.0 % for 30 min was effective in killing both vegetative and zoosporic stages of the fungal strain.

Toxicity tests were conducted using Asian seabass and Nile tilapia, *Oreochromis niloticus* (Linnaeus 1758) fry with the same treatment levels of seawater and NaCl for up to 2 h and mortality was recorded at 24 h. No mortality was observed in any of the Asian seabass fry groups, but 100 % mortality was observed for Nile tilapia fry in 3.0 % NaCl for 1 and 2 h treatments. Use of seawater and NaCl in aquaculture was effective for controlling a pathogenic *Achlya* sp. strain, but toxicity to the target aquaculture species needs to be assessed to determine a treatment concentration and duration that would ensure effectiveness as a fungicide as well as to minimise toxicity to the fish fry.

Keywords: Seawater, sodium chloride, hyphal growth, zoospores germination, *Achlya*, Asian seabass, Nile tilapia

[*] Corresponding author. E-mail address: khatai0111@nvlu.ac.jp

Introduction

Achlya infection is an aquatic fungal disease that frequently develops at all life stages of freshwater fishes (Hussein et al. 2002). The genus *Achlya* of the family Saprolegniaceae (Bruno et al. 2011) is ubiquitous in natural water sources near local fish farms and hatcheries, especially in tropical countries. Outbreaks of this infection have been implicated in mass mortality of cultured fish and continue to be a serious disease problem, causing great losses in aquaculture production (Noga 1996; Chukanhom and Hatai 2004).

Treatment of this infection is very difficult due to limitations of legally available chemicals (Zahran and Risha 2013). In the past, malachite green and formalin were widely used as effective fungicides. However, malachite green was discontinued for fungal infection control in aquatic animals reared for food due to its toxicity, mutagenicity and carcinogenicity to cultured animals and humans (Treves-Brown 2000; Sudova *et al*. 2007; Fuangsawat *et al*. 2011). Use of formalin had a negative impact on the environment as well as administering personnel (Fitzpatrick *et al*. 1995), due to its resilience in aquatic ecosystems and detrimental effect on human health (Schreier *et al*. 1996). Hydrogen peroxide (H_2O_2), sodium chloride (NaCl) and potassium permanganate ($KMnO_4$) have been reported to be effective antifungal chemicals in aquaculture (Marking *et al.* 1994; Hanjavanit *et al*. 2013) and have been recommended to prevent *Achlya* infection. Apart from these chemical remedies, plant herbs have also been studied as potent anti-oomycete agents against *Achlya* infection (Panchai *et al*. 2015). However, the use of plant herbs has been very limited due to their low percentage yield and availability (Cheeptham and Towers 2002), and the toxicity of these plants limits their efficacy (Mori *et al.* 2002).

Therefore, studies on alternative, more easily available antifungal agents with less impact on aquatic animals, human health and the environment are necessary for improving fungal infection control. Treatment of *Achlya* infection has been widely accomplished using therapeutic or prophylactic administration of chemicals (Borisutpeth *et al.* 2010). However, the use of chemicals poses difficulties due to food safety issues and consumer health concerns regarding fish for human consumption (Schreier *et al.* 1996).

As *Achlya* infects Asian seabass, *Lates calcarifer* (Bloch 1790) only when it is reared in freshwater, it was therefore thought that seawater should be effective to control fungal infection in this species. In addition, NaCl, which is the major compound of seawater, was also studied as a comparison for its effectiveness against *Achlya* infection. The aims of the present study were to examine i) the antifungal activity of seawater and NaCl on hyphae and zoospores of *Achlya* sp. IPMB 1403 and ii) toxicities of seawater and NaCl to freshwater-reared Asian seabass, *L. calcarifer* and Nile tilapia, *Oreochromis niloticus* (Linnaeus 1758) fry.

Materials and Methods

Fungal strain

The isolated fungal strain *Achlya* sp. IPMB 1403 was kept at the Microbiology and Fish Disease Laboratory, Borneo Marine Research Institute and routinely maintained on glucose yeast extract (GY) agar (1.0 % glucose, 0.25 % yeast extract and 1.5 % agar) (Hatai and Egusa 1979) at 25 °C and sub-cultured to fresh GY agar monthly.

The advancing edges of 7-d growing hyphal colonies of the isolated fungal strain on GY agar were cut from the agar block using a No. 2 cork borer (5.5 mm in diameter) and used as inoculums for all experiments.

Fungistatic effects of seawater and NaCl on hyphal growth

The agar block cores were each inoculated onto the centre of Petri dishes (80 x 20 mm) with 20 mL GY agar containing 0 (control), 5, 10, 15 or 20 ppt seawater or 0 (control), 0.5, 1.0, 1.5, 2.0, 2.5 or 3.0 % NaCl and then incubated at 25 °C. At 3, 5, 7 and 10 d after inoculation, two diameters of the hyphal colony measured at right angles to each other using Vernier calipers according to Borisutpeth *et al*. (2014) were used to calculate the mean diameter:

$$R = \frac{HC-AB}{2}$$, where:

R = radius of hyphal colony (mm); HC = mean diameter of hyphal colony (mm);

AB = mean diameter of circular agar block (mm)

Fungicidal effects of seawater and NaCl on hyphal growth

Three agar-block cores cut out from the edge of the parent colony were exposed to seawater (10, 20 and 30 ppt) or NaCl solution (1.0, 2.0 and 3.0 %) for 30 min, 1 and 2 h, and sterile tap water (STW) without seawater or NaCl as the control. After various exposure times, one agar block was removed, rinsed in STW and inoculated on GY agar. Hyphal growth was observed by eye after incubation at 25 °C for 24 h.

Fungicidal effects of seawater and NaCl on zoospore germination

Hyphae grown in GY broth for 3 d were inoculated into STW at 25 °C for 24 h for zoospore production (Kitancharoen and Hatai 1996). Zoospore number in the zoospore suspension was adjusted to 1.0 x 10^4 spores.mL^{-1} (Hanjavanit *et al.* 2013). A 1 mL suspension of zoospores was inoculated into test tubes containing 9 mL of seawater at 0 (control), 10, 20 and 30 ppt or NaCl solution at 0 (control), 1.0, 2.0 and 3.0 %.

At 30 min, 1 and 2 h, 0.1 mL of solution from each test tube was taken for inoculation in 10 mL of GY broth. Zoospores germination was determined by visual observation after incubation at 25 °C for 48 h.

Source of experimental fish

Healthy freshwater-reared Asian seabass fry (average body weight (BW) 2.5 g; and average total length (TL), 3.5 cm) and Nile tilapia fry (average BW 3.2 g; average TL 5.4 cm) were obtained for the toxicity test.

The experimental fish were acclimatised in dechlorinated water with aeration at 25 °C for 1 week. The fish were fed with commercial formula food twice a day and starved for one day before the experiment (Ellsaesser and Clem 1986).

Toxicity effects of seawater and NaCl on Asian seabass or Nile tilapia fry

The experiments were divided into three trials as follows: i) The control group in STW (0 ppt seawater or 0 % NaCl); ii) experimental group I in 10, 20 and 30 ppt seawater; and iii) the experimental group II in 1.0, 2.0 and 3.0 % NaCl. Fifteen fish fry of each group were placed in 5 L of the respective solution with aeration at 26-28 ºC. At 30 min, 1 and 2 h, five fish fry were removed from each treatment, rinsed and placed into STW with aeration. The number of fish surviving at 24 h was used to calculate the corrected mortality following Abbott's formula (Barnes *et al*. 1998):

$$\text{Pt} = \frac{(Po - Pc)}{(100 - Pc)}\ x\ 100, \text{ where:}$$

Pt = percentage of corrected mortality (%); Po = mortality of test group; Pc = mortality of control group.

Results

Fungistatic effects of seawater and NaCl on hyphal growth

In vitro hyphal growth of *Achlya* sp. IPMB 1403 was completely inhibited when exposed to seawater at concentrations of 15 and 20 ppt. NaCl completely inhibited hyphal growth at concentrations of 1.5, 2.0, 2.5 and 3.0 % (Table 1).

Table 1. Fungistatic effects of seawater and NaCl on hyphal growth

	Concentration	Mean radius of hyphal colony (mm)			
		3	5	7	10
Control: without seawater or NaCl	0	8.50	13.50	22.50	36.50
Seawater (ppt)	5	7.75	12.25	15.25	19.75
	10	5.75	10.25	12.75	16.25
	15	0	0	0	0
	20	0	0	0	0
NaCl (%)	0.5	7.25	11.25	13.50	17.50
	1.0	1.25	1.25	1.25	1.25
	1.5	0	0	0	0
	2.0	0	0	0	0
	2.5	0	0	0	0
	3.0	0	0	0	0

Fungicidal effects of seawater and NaCl on hyphal growth

As shown in Table 2, *Achlya* sp. IPMB 1403 grew in seawater of various salinities (10, 20 and 30 ppt) during all immersion periods but only grew in 2.0 % NaCl for up to 1 h. Therefore, the effective fungicidal dose of NaCl on hyphal growth of the fungal strain was 2.0 % for a 2 h treatment and 3.0 % for 30 min. Slow growth was observed when exposed to 30 ppt seawater for 2 h and 2.0 % NaCl for 1 h.

Table 2. Fungicidal effects of seawater and NaCl on hyphal growth at 25 °C

Immersion period	Hyphal growth*							
	Seawater (ppt)				NaCl (%)			
	0	10	20	30	0	1.0	2.0	3.0
30 min	++	++	+	+	++	+	+	-
1 h	++	++	+	+	++	+	+	-
2 h	++	++	+	+	++	+	-	-

* -, no growth; +, slow growth; or ++, rapid growth

Fungicidal effects of seawater and NaCl on zoospore germination

As shown in Table 3, zoospore germination of *Achlya* sp. IPMB 1403 showed similar results for exposure to seawater and NaCl solution. Seawater and NaCl concentrations with a fungicidal effect on zoospore germination were at 20 ppt seawater and 2.0 % NaCl for 2 h treatment. Zoospores of the fungal strain could not germinate following contact with 30 ppt seawater and 3.0 % NaCl.

Table 3. Fungicidal effects of seawater and NaCl on hyphal growth from zoospore at 25°C

Immersion period	Hyphal growth*							
	Seawater (ppt)				NaCl (%)			
	0	10	20	30	0	1.0	2.0	3.0
30 min	+	+	+	-	+	+	+	-
1 h	+	+	+	-	+	+	+	-
2 h	+	+	-	-	+	+	-	-

*: -, no growth; or +, growth

Toxicity effects of seawater and NaCl on Asian seabass and Nile tilapia fry

Toxicity effects of seawater and NaCl solutions at concentrations that showed inhibition of hyphal growth and zoospore germination of *Achlya* sp. IPMB 1403 were tested with Asian seabass and Nile tilapia fry. The mortality of fish fry at 24 h after exposure with different treatments is shown in Table 4. While Asian seabass fry showed no mortality at 24 h for all treatment and control groups, Nile tilapia fry showed mortality for longer exposure at higher concentrations. Nile tilapia fry showed 100 % mortality at 24 h following exposure to 3.0 % NaCl for 1 and 2 h and 20 % mortality following exposure to 20 and 30 ppt seawater for 2 h, 2.0 % NaCl for 2 h and 3.0 % NaCl for 30 min.

Table 4. Mortality data for Asian seabass fry/Nile tilapia fry at each treatment level and exposure duration

Exposure duration	Mortality (%)							
	Seawater (ppt)				NaCl (%)			
	0	10	20	30	0	1.0	2.0	3.0
30 min	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 20
1 h	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 100
2 h	0 / 0	0 / 0	0 / 20	0 / 20	0 / 0	0 / 0	0 / 20	0 / 100

Discussion

The present study showed that NaCl effectively inhibited and killed both hyphae and zoospores of *Achlya* sp. IPMB 1403, while seawater only decreased the growth of hyphae. Hanjavanit *et al.* (2013) reported a minimum inhibitory concentration (MIC) of 2.5 % for NaCl against hyphal growth of an isolate of *Achlya* sp. In our study, the fungistatic dose that inhibited growth was 15 ppt seawater or 1.5 % NaCl, and the fungicidal activity on hyphal growth and zoospore germination was achieved with 2.0 % NaCl for 2 h while 3.0 % NaCl was toxic after 30 min.

These results are similar to reports of Marking *et al.* (1994) and Hanjavanit *et al.* (2013) in which the vegetative and zoosporic stages of the *Achlya* sp. isolates tested were effectively killed with 3.0 % NaCl for 1 h or 2.5 % NaCl for 2 h, respectively. However, Khodabandeh and Abtahi (2006) reported that 3.5 % NaCl was most effective for controlling aquatic fungal infection. For tests with seawater in our study, hyphae survived dipping in seawater for all durations tested (up to 3 h) and zoospores did not germinate after exposure to 20 ppt seawater for 2 h and 30 ppt seawater for all dipping times (up to 3 h).

This difference in survival may be due to the difference in sensitivity with zoospores being more sensitive to chemicals than hyphae, according to Muller-Breban *et al*. (1995). Survival of Asian seabass and Nile tilapia fry exposed to seawater and NaCl was taken into consideration for establishing effective treatment for *Achlya* infection. In the toxicity test, no mortality occurred in Asian seabass fry in any group (seawater, NaCl or control) during the immersion period. However, Nile tilapia fry could not tolerate high concentrations of NaCl (3.0 %), and 100 % mortality resulted if exposure was longer than 1 h. Immersion in seawater at 20 ppt for 2 h, 3.0 % NaCl for 30 min or 2.0 % NaCl for 2 h was highly effective against hyphal growth and zoospore germination of pathogenic strain *Achlya* sp. IPMB 1403 *in vitro* and was tolerated by Asian seabass fry.

For aquaculture of Nile tilapia fry, treatments at 30 ppt seawater for 2 h or 3.0 % NaCl for 30 min were effective for controlling *Achlya* and were tolerated by the fry. In previous studies, NaCl was widely studied as an effective incubation treatment for freshwater fish eggs (Marking *et al*. 1994; Schreier *et al.* 1996; Khodabandeh and Abtahi 2006; Rasowo *et al*. 2007; Hanjavanit *et al.* 2013), but studies did not include fish fingerlings or fry. The present study showed that seawater and NaCl are safe and efficacious for routine use in treating fish fry reared in freshwater to prevent the occurrence of *Achlya* infection.

Conclusion

This study is the first report on the effects of seawater and NaCl on hyphal growth and zoospore germination of the pathogenic fungus *Achlya* sp. IPMB 1403, which was isolated from Asian seabass fry. NaCl was more effective than seawater for controlling *Achlya* infection in both seabass and tilapia fry. Although they differ in terms of their effectiveness, both treatments are easily available, pose low risks to personnel and the environment, and are not costly when compared to other effective antifungal treatments (Marking *et al*. 1994). The use of NaCl may thus be a good approach for freshwater aquaculture facilities and rural fish farmers, as it is an easy, immediate and effective method for preventing *Achlya* infection in freshwater-reared fishes. Apart from NaCl, seawater may also be recommended for routine use to treat massive numbers of freshwater fish fry in marine multi-species hatcheries.

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