Mycoscience 38: 375–378, 1997

Fungicidal effect of hydrogen peroxide on fungal infection of rainbow trout eggs

Nilubol Kitancharoen[1], Atsushi Yamamoto[2] and Kishio Hatai[1]

[1] Division of Fish Diseases, Nippon Veterinary and Animal Science University, 1–7–1, Kyonan-cho, Musashino, Tokyo 180, Japan

[2] Oshino Trout Hatchery, Yamanashi Prefectural Fisheries Technology Center, Oshino, Yamanashi 401–05, Japan

Accepted for publication 15 September 1997

Exposure to 1,500 μg/ml of hydrogen peroxide (H_2O_2) for 60 min at 13°C was found to be injurious to rainbow trout eggs. On the other hand, the concentration which effectively inhibited pathogenic fungi in vitro was substantially less than this toxic dosage; specifically, 500 μg/ml for 60 min at 20°C to inhibit the zoosporic stage and 1,000 μg/ml for 60 min at 20°C to inhibit the vegetative stage. From in vivo tests, treatment with 1,000 μg/ml of H_2O_2 for 60 min at 13°C was found to be the most effective procedure to control fungal infection and increase the hatching rate of rainbow trout eggs.

Key Words——fungicidal activity; hatching rate; hydrogen peroxide; rainbow trout egg.

Aquatic fungi, which are ubiquitous in the water systems of hatcheries, often cause serious damage to incubating eggs, particularly when prophylactic treatment with chemicals is not applied. Malachite green is commonly used to solve this problem and has been widely reported as an effective fungicidal agent (Cline and Post, 1972; Bailey, 1984; Alderman, 1985; Li et al., 1996). Unfortunately, in the 1991 FDA registration, malachite green is prohibited for use in aquaculture because of its teratogenic properties and the fact that the effects of its residues are still obscure (Meyer and Jorgenson, 1983; Meinertz et al., 1995). Edgell et al. (1993) reported that malachite green treatment induced a higher incidence of yolk-sac abnormality in alevin than salt treatment. Many attempts have been made to find an alternative fungicidal agent. Hydrogen peroxide (H_2O_2), previously used for parasitic control in fish, is one of the chemicals which has been recommended for fungal prevention (Dawson et al., 1994).

In the present study, we focused on H_2O_2 because it has no known harmful effects on fish. It is naturally reduced to H_2O and O_2, which are not hazardous. Furthermore, few works have evaluated the possibility of using this chemical as a fungicidal agent on fish eggs.

Materials and Methods

Fungi The fungi used in the present study are detailed in Table 1. The fungi were cultured on GY agar to obtain the vegetative stage, and in GY broth before transfer to sterilized tap water at 10°C to obtain the zoosporic stage, as described by Kitancharoen et al. (1996). To obtain numerous zoospores, the fungus was kept in sterilized tap water at 10°C for 48–72 h.

Toxicity of H_2O_2 to rainbow trout eggs The minimum concentration of H_2O_2 which reduced the survival of rainbow trout eggs was verified to determine the proper dosages for treatment. Eyeing eggs of rainbow trout were prepared in groups of 50 eggs. The eggs were bathed in various concentrations of H_2O_2 (Mitsubishi Gas Chemical, 31% active ingredient), as shown in Table 2, for 60 min. The experiment was carried out in 500-ml plastic cups containing 400 ml of H_2O_2 solution. Aeration was provided during this time. Thereafter, the eggs were transferred to plastic mesh baskets (about 10 cm in diam) in a 50-L plastic tank with running water. Water temperature during the experiment was 13°C. The test was performed in duplicate. The mortality was determined after 72 h.

Fungicidal activity of H_2O_2 The three concentrations of H_2O_2 (250, 500 and 1,000 μg/ml) which appeared to have no toxicity to rainbow trout eggs were tested for their fungicidal activities against the vegetative stage and against the zoosporic stage.

Activity against the vegetative stage was investigated with fungal strains cultured on 20-ml GY agar plates for 2–3 d at 20°C. An active part of each strain was excised with a no. 1 cork borer (4.5 mm in diam) and placed into H_2O_2 solution for 30 or 60 min at 20°C, then washed twice with 50 ml of sterilized distilled water and placed onto a 20-ml GY agar plate. The activity of H_2O_2 against the vegetative stage was determined by comparing colony diameter (mm) of the treated strains with a control which had been immersed in sterilized tap water. When no growth appeared after 24 h at 20°C, the plates were kept for 3 d to observe fungal viability.

Activity against the zoosporic stage was estimated with zoospores prepared in sterilized tap water at 10°C,

Table 1. Fungal isolates used in the present study.

Fungus	Location (Pref.)	Host (egg)[a]	Date
Saprolegnia ferax (Gruith.) Thuret			
NJM 9580	Shizuoka	rainbow trout	Jul. 95
S. diclina Humphrey			
NJM 9585	Shizuoka	rainbow trout	Jul. 95
S. parasitica Coker			
NJM 9595	Yamanashi	sockeye salmon	Oct. 95
S. hypogyna (Pringsh.) de Bary			
NJM 9668	Tochigi	masu salmon	Nov. 96
Achlya sp.			
NJM 9598	Yamanashi	rainbow trout	Dec. 95
Aphanomyces frigidophilus Kitancharoen & Hatai			
NJM 9500	Tochigi	Japanese char	Dec. 95
Pythium monospermum Pringsh.			
NJM 9659	Okutama	rainbow trout	Apr. 95
Leptolegnia sp.			
NJM 9671	Nikko	honmasu	Nov. 96

a) Scientific names: rainbow trout, *Oncorhynchus mykiss* (Walbaum); sockeye salmon, *O. nerka* (Walbaum); Japanese char, *Salvelinus leucomaenis* (Pallas); masu salmon and honmasu, *O. masu* (Brevoort).

as described by Kitancharoen et al. (1996). Zoospores were harvested by aseptic filtration through gauze, their number was evaluated using an improved Neubauer chamber (Erma R), and suspensions were prepared containing 2×10^4 spores/ml. Then 1 ml of H_2O_2 solution of 10 times the desired final concentration was added to 9 ml of zoospore suspension, and the mixture was left to stand for 30 or 60 min. Then a 120-μl portion of the mixture was inoculated into 40 ml of GY broth. A preliminary test has shown that this dilution ratio had no affect on the germination of viable spores. The viability of zoospores was determined by observing the appearance of germinating thalli during 3 d under microscope. A control experiment without H_2O_2 treatment was also performed. The experiments were performed at 20°C in duplicate.

Repeated treatment with H_2O_2 of incubating rainbow trout eggs Rainbow trout eggs obtained from two 4-yr-old females were inseminated, held for about 1 h, to allow hardening, then randomly grouped into batches of 800 and held in plastic mesh baskets. The eggs were treated with 500 or 1,000 μg/ml of H_2O_2 at 13°C, these concentrations having been shown to cause no injury to rainbow trout eggs and to be effective to control fungi. Treatment was performed in 10-L tanks containing 2 L water with aeration for 60 min, and it was repeated twice a week until the eggs hatched out. Between treatments, the eggs were held in a 50-L tank with running water at 13°C. A control group was manipulated in the same manner, but without H_2O_2. The flow rate of water was about 18 ml/min. The number of eggs with fungal infection and the hatching rate were determined on day 35.

Table 2. Mortality of rainbow trout eggs after immersion in various concentrations of H_2O_2 for 60 min at 13°C.

H_2O_2 concentration (μg/ml)	Mortality (%means±S.D.)
250	0
500	0
1,000	0
1,500	30.0±2.0
2,000	48.0±4.0
2,500	71.0±3.0
Control (without H_2O_2)	0

Results

Toxicity of H_2O_2 to rainbow trout eggs Treatment of rainbow trout eggs with various concentrations H_2O_2 for 60 min at 13°C demonstrated that concentrations between 250 μg/ml and 1,000 μg/ml were not toxic to the eggs (Table 2). Exposure to 1,500 μg/ml of H_2O_2 for 60 min appeared to be toxic to rainbow trout eggs, leading to 30.0±2.0% mortality rate.

Fungicidal activity of H_2O_2 As shown in Table 3, treatment with 1,000 μg/ml of H_2O_2 for 60 min at 20°C appeared to be effective to kill the vegetative stage of all fungal strains used. Treatment with the lower concentration of H_2O_2 (250 μg/ml), even for 30 min, retarded the growth of the fungi to less than half of the control group, except in *Saprolegnia hypogyna* NJM 9668. On exposure for 30 min, *Achlya* sp. NJM 9598 and *Pythium monospermum* NJM 9659 showed higher tolerances to H_2O_2 than the other fungi, even when exposed to 1,000 μg/ml of H_2O_2. Treatment with H_2O_2 at the concentration of 500 μg/ml for 60 min (Table 4), inhibited zoospore germination of all isolates. The zoospores of the strains belonging to the genus *Saprolegnia* (except *S.*

Table 3. Effects of dosages and exposure times of H_2O_2 on vegetative growth of the tested fungi.

Conc.	Exposure time	*S. ferax* NJM 9580	*S. diclina* NJM 9585	*S. parasitica* NJM 9595	*S. hypogyna* NJM 9668	*Achlya* sp. NJM 9598	*A. frigidophilus* NJM 9500	*P. monospermum* NJM 9659	*Leptolegnia* sp. NJM 9671
Control	30 min	31.1[a]	24.7	19.8	15.6	17.8	6.9	8.5	12.0
	60 min	30.2	25.3	19.8	14.6	17.8	6.7	8.3	11.8
250 μg/ml	30 min	+[b]	8.1	7.7	11.9	7.1	−[c]	8.5	6.3
	60 min	−	7.8	+	7.6	5.6	−	7.2	−
500 μg/ml	30 min	−	+	+	+	5.3	−	7.1	−
	60 min	−	−	−	−	−	−	5.0	−
1,000 μg/ml	30 min	−	−	−	−	+	−	5.8	−
	60 min	−	−	−	−	−	−	−	−

a) Colony diameter (mm) after incubation at 20°C for 24 h.
b) +: Viability retained and vegetative growth appeared after 24 h.
c) −: No vegetative growth detected within 24 h.

Table 4. Effect of dosages of H_2O_2 and exposure times at 20°C on zoospore germination of the tested fungi.

Conc.	Exposure time	*S. ferax* NJM 9580	*S. diclina* NJM 9585	*S. parasitica* NJM 9595	*S. hypogyna* NJM 9668	*Achlya* sp. NJM 9598	*A. frigidophilus* NJM 9500	*P. monospermum* NJM 9659	*Leptolegnia* sp. NJM 9671
Control	30 min	+[a]	+	+	+	+	+	+	+
	60 min	+	+	+	+	+	+	+	+
250 μg/ml	30 min	+	+	+	+	−[b]	−	−	+
	60 min	−	+	+	+	−	−	−	−
500 μg/ml	30 min	+	+	+	+	−	−	−	+
	60 min	−	−	−	−	−	−	−	−
1,000 μg/ml	30 min	−	+	−	+	−	−	−	−
	60 min	−	−	−	−	−	−	−	−

a) +: Germination detected within 72 h observation.
b) −: No germination detected within 72 h observation.

parasitica NJM 9595) and of *Leptolegnia* sp. NJM 9671 showed higher tolerance to H_2O_2 than their vegetative stages on exposure for 30 min. On the other hand, the zoospores of *Achlya* sp. NJM 9598 and of *P. monospermum* NJM 9659 were apparently more sensitive to H_2O_2 than their vegetative stages. *Aphanomyces frigidophilus* NJM 9500 had high sensitivity to H_2O_2 in both stages.

Repeated treatment with H_2O_2 of incubating rainbow trout eggs Repeated treatment with the three concentrations of H_2O_2 for 60 min at 13°C significantly reduced fungal infection and improved the hatching rate of rainbow trout eggs. The concentration of 1,000 μg/ml was the most effective to control fungal infection. As the

Table 5. Repeated treatment with H_2O_2 for 1 h on fungal control and hatching rate of rainbow trout eggs.

H_2O_2 (μg/ml)[a]	Percentage of fungal infected eggs after 35 d incubation (%means±S.D.)	Hatching rate (%means±S.D.)
250	59.2±5.4	37.4±2.7
500	40.7±2.7	46.6±4.3
1,000	28.9±8.8	67.6±2.8
Control	92.2±3.4	7.8±3.3

a) Treated at 13°C for 60 min, twice a week.

results in Table 5, treatment with 1,000 μg/ml of H_2O_2 significantly improved the hatching rate by about 20% over treatment with 500 μg/ml of H_2O_2, and about 30% over treatment with 250 μg/ml of H_2O_2.

Discussion

Hydrogen peroxide (H_2O_2), an antimicrobial agent, is used in aquaculture to treat external parasites such as protozoa, monogenea and fish lice. It is also effective to control fungal infection on fish and fish eggs. The present study has confirmed both in vitro and in vivo that it is effective for fungal control. H_2O_2 treatment is a practical method that can be performed in the hatchery system. The present study confirmed the finding of Marking et al. (1994) that the toxic dosage of H_2O_2 to rainbow trout was from 1,500 μg/ml. From our results, treatment with 500 μg/ml H_2O_2 for 60 min at 13°C was effective to control the zoospores of all tested fungi, but this treatment could not inhibit the vegetative growth of some isolates. In hatcheries, infection by vegetative hyphae is common. Thus, as a practical procedure, we suggest treatment with 1,000 μg/ml of H_2O_2 for 60 min at 13°C which was effective to control infection by both stages of the fungi.

Marking et al. (1994) reported that concentrations of 500–1,000 μg/ml of H_2O_2 were effective to control fungal infection by an isolate of *S. diclina*, on rainbow trout eggs at 12°C. The present study demonstrated that the same concentrations were effective to control infections by fungi which occur naturally on rainbow trout eggs. Dawson et al. (1994) proposed that treatment with 500 μg/ml of H_2O_2 for 15 min every other day would inhibit fungal infection on rainbow trout eggs which were not previously infected. However, an exposure time of 60 min was necessary to control fungal infection on 10% infected eggs. From our results, we recommend that, in addition to routine screening of dead eggs, treatment with H_2O_2 is successful to improve the fungal infection control and increase the hatching rate of rainbow trout eggs.

Literature cited

Alderman, D. J. 1985. Malachite green: a review. J. Fish Dis. **8**: 289–298.

Bailey, T. A. 1984. Effects of twenty-five compounds on four species of aquatic fungi (Saprolegniales) pathogenic to fish. Aquaculture **38**: 97–104.

Cline, T. F. and Post, G. 1972. Therapy for trout eggs infected with *Saprolegnia*. Prog. Fish-Cult. **34**: 148–151.

Dawson, V. K., Rach, J. J. and Schreier, T. M. 1994. Hydrogen peroxide as a fungicide for fish culture. Bull. Aquacul. Assoc. Canada **2**: 54–56.

Edgell, P., Lawseth, D., McLean, W. E. and Britton, E. W. 1993. The use of salt solutions to control fungus (*Saprolegnia*) infestations on salmon eggs. Prog. Fish-Cult. **55**: 48–52.

Kitancharoen, N., Yuasa, K. and Hatai, K. 1996. Effects of pH and temperature on growth of *Saprolegnia diclina* and *S. parasitica* isolated from various sources. Mycoscience **37**: 385–390.

Li, M. H., Wise, D. J. and Robinson, E. H. 1996. Chemical prevention and treatment of winter saprolegniosis ("winter kill") in channel catfish *Ictalurus punctatus*. J. World Aqua. Soc. **27**: 1–6.

Marking, L. L., Rach, J. J. and Schreier, T. M. 1994. Evaluation of antifungal agents for fish culture. Prog. Fish-Cult. **56**: 225–231.

Meinertz, J. R., Stehly, G. R., Gingerich, W. H. and Allen, J. L. 1995. Residues of [^{14}C]-malachite green in eggs and fry of rainbow trout, *Oncorhynchus mykiss* (Walbaum), after treatment of eggs. J. Fish Dis. **18**: 239–247.

Meyer, F. P. and Jorgenson, T. A. 1983. Teratological and other effects of malachite green on the development of rainbow trout and rabbits. Trans. Amer. Fish. Soc. **112**: 818–824.