Bull. Fac. Bioresources, Mie Univ.
No.33 : 1−7
March 31, 2006

Fungicidal Activities of Horseradish Extract on a Fish-Pathogen Oomycetes, *Saprolegnia parasitica*

Chutima KHOMVILAI*, Masaaki KASHIWAGI and Motoi YOSHIOKA**
Faculty of Bioresources, Mie University, 1577, Kurimamachiya-cho,Tsu, Mie 514-8507, Japan

Abstract

To evaluate the antifungal activities of horseradish *Armoracia rusticana* extract, the mycelia and zoospores of *Saprolegnia parasitica* NJM 8604 were treated with various combinations of allyl isothiocyanate (AIT) concentration and exposure times. AIT is a main pungent component of horseradish. The minimum inhibitory concentration for mycelial growth was 68 mg/L with 60-min exposure and that for zoospore germination was 42.5 mg/L with 5-min exposure. These values were significantly lower than those reported in previous research. The horseradish extract is an effective antifungal agent against saprolegniasis.

Key Words: allyl isothiocyanate, antifungal activity, horseradish extract, *Saprolegnia parasitica*

Introduction

Aquatic fungi of the order Saprolegniales often cause serious damage to freshwater fishes if no effective prophylactic or therapeutic treatments are applied to them[1]. Malachite green is quite an effective antifungal agent, but it is teratogenic[2], residual[3]and carcinogenic[4]. In Japan, the Pharmaceutical Affairs Law prohibited the use of it in 2003. The caretaker extension time for malachite green had just expired in July 2005. An alternative antifungal agent is needed to be effective and safe for non-target organisms as well as the environment. Current researches for alternative agents have focused mainly on three candidates, formalin[5-8], hydrogen peroxide[6-11], and sodium chloride or seawater[6-12]. These are effective, but have some negative points. For example, formalin is potentially harmful to the user's health and remains in the environment. Regarding hydrogen peroxide, the undiluted solution is strongly corrosive and combustible. And, as its effective concentration is required to be as high as 1,000 mg/L, it has not been permitted in the USA[6]. Sodium chloride, in spite of its safety, may be limited in its applicability due to the high cost of acquiring effective concentrations as high as 30,000 mg/L.

Up to now, we have demonstrated that sodium hypochlorite has a potential to act as an alternative agent that is superior in both safety and affordability to the fore mentioned chemicals[13-20]. Furthermore, we have been trying to search for safer agents; not synthetic chemicals but near-natural substances. Under these parameters, we obtained the extract of horseradish *Armoracia rusticana*, which is commonly used as a spice

Accepted: March 10. 2006

* Present address; Department of Fisheries, Kaset-Klang, Chatvchak, Bangkok 10100, Thailand

** For correspondence (e-mail: motoi@bio.mie-u.ac.jp)

for Japanese and Chinese foods. It is well known that allyl isothiocyanate (AIT; CH_2=CH-CH_2-N=C=S), being a main pungent component of horseradish, wasabi *Wasabia japonica*, brown mustard *Brassica juncea* and black mustard *B. nigra* shows powerful bactericide[21-23] and fungicide[24], and has also been proven to have some positive effects on human health such as respiratory diseases[25]. Hence, we observed the fungicidal activities of AIT against *Saprolegnia parasitica*, a fish-pathogen oomycetes, and determined both minimum inhibitory concentrations (MICs) for mycelial growth and zoospore germination. Incidentally, although Mori *et al.*[26] already gave both MICs for the same fungus, we chose to report our own experimental results which were significantly different from their values, because both concentration and exposure time are important factors in safety and cost analysis for practical use of AIT as an antifungal agent.

Materials and Methods

Horse radish extract

A brand name of horseradish extract hot oil (Yamachu Wasabi Co. Ltd.) was used in this study. The extract includes 85% of AIT as its main ingredient. To prepare the test concentrations of AIT solution, the extract oil was emulsified to be a water soluble compound, including 8.5% of AIT, and then it was diluted with distilled water to each target concentration.

Fungus

Fungus used in this study was a pure strain NJM 8604 of *Saprolegnia parasitica* Coker that was isolated from cultured coho salmon *Oncorhynchus kisutch*. Professor K. Hatai, Nippon Veterinary and Animal Science University kindly supplied it. In our laboratory, the fungus had been maintained on glucose-yeast (GY) agar and refreshed every 3 or 4 weeks at 5℃[27].

Mycelial growth inhibitory test

Preliminarily, to decide the test incubation temperature for the fungus, the agar plugs were cut out with a cork borer 5.5 mm in diameter from the edge of active growing colony, and placed on the center of two experimental GY agar plates, then incubated for 2 days at 5, 10, 15, and 20℃. Consequently, the fungal growth was better at the latter two temperatures than at the former two, and there was no statistical difference between the latter two (Table 1). Accordingly, the former temperature, being suitable for salmon, was used in this study. Next the agar plugs were cut out in the same manner as described above, and put into 1,000 mL of AIT solution at concentrations of 68, 85, and 102 mg/L with 30-min and 60-min exposures. After this, each plug was rinsed twice with 50 mL of sterile tap water and placed on the center

Table 1 Colony diameters (mm) of *Saprolegnia parasitica* at various incubation temperatures

5℃	10℃	15℃	20℃
11.5 ± 0.2^a	28.2 ± 4.5^b	53.7 ± 1.3^c	61.5 ± 1.2^c

The different letters on the upper right side of each colony diameter indicate a significant difference between treatments (Duncan's new multiple range test, $p<0.05$)

of two experimental GY agar plates, and incubated for 2 days at 15℃.

The inhibitive activity was evaluated by comparing colony diameter between AIT treatments and the untreated controls, and is represented as the colony diameter growth rate calculated from the equation; (colony diameter of the treated fungus)/(colony diameter of the control) × 100 (%). These data were subjected to one-way analysis of variance followed by Duncan's new multiple range test to identify differences among means at $p<0.05$.

Zoospore germination inhibitory test

For zoospore preparation, two agar pieces, 8 × 8 mm in size were cut out from the edge of an active growing colony and incubated in a 500 mL GY broth for 6 days at 15℃. The mass of grown mycelia was then rinsed three times with sterile tap water and kept in 500 mL of sterile tap water for 3 days. As sporangia producing zoospores developed during this period, the zoospores in suspensions were obtained by aseptic filtration through gauze.

The zoospores were put into 1,000 mL of AIT solution at 34, 42.5, and 51 mg/L with 10-s, 1-min and 5-min exposures. The density of zoospores was adjusted to 2×10^5 spores/1000 mL by the Bürker Türk haemocytometer (Bürker-Türk, Emergo, Landsmere, the Nederland). Following this, 1 mL from each mixture containing the treated zoospores was put into four Petri-dishes with three hemp seeds and 30 mL of sterile tap water, then incubated for 5 days at 15℃.

The inhibitive activity was evaluated by observing whether the zoospores germinated on the hemp seeds or not.

Results

Inhibitive activity on mycelial growth

The experimental results are shown in Table 2. Colony diameter of the control was around 50 mm for all exposure times. While the mycelia exposed to AIT did not grow at all, except for a trial at 68 mg/L with 30-min exposure. According to the Duncan's new multiple range test, there were statistically significant differences between AIT and the control ($p<0.05$). Accordingly, the minimum inhibitive concentration (MIC) was determined to be 68 mg/L with 60-min exposure.

Table 2 Colony diameters (mm) of *Saprolegnia parasitica* after allyl isothiocyanate (AIT) treatments at various combinations of AIT concentration and exposure time.

AIT (mg/L)	Exposure Time	
	30 min	60 min
0	54.1 ± 0.4^a	53.3 ± 0.2^a
68	19.6 ± 2.5^b	0 ± 0^c
85	0 ± 0^c	0 ± 0^c
102	0 ± 0^c	0 ± 0^c

The different letters on the upper right side of each colony diameter indicate a significant difference between treatments (Duncan's new multiple range test, $p<0.05$)

Table 3 Zoospore germination (+, positive: −, negative) of *Saprolegnia parasitica* after allyl isothiocyanate (AIT) treatments at various combinations of AIT concentration and exposure time.

AIT (mg/L)	Exposure Time		
	10 s	1 min	5 min
0	+++ +++ +++ +++	+++ +++ +++ +++	+++ +++ +++ +++
34	+++ +++ +++ −−−	+++ +++ +++ +++	+++ +++ +++ +++
42.5	+++ +++ +++ +++	+++ −−− +−− −−−	−−− −−− −−− −−−
51	−−− −−− −−− −−−	−−− −−− −−− −−−	−−− −−− −−− −−−

Each symbol indicates one hemp seed, and a group of three symbols shows an experimental dish with three hemp seeds.

Inhibitive activity on zoospore germination

The experimental results are shown in Table 3. Germinated hyphae were observed on all hemp seeds of the controls, but not on those of AIT treatments at 42.5 mg/L with 5-min exposure and 51 mg/L with all exposure times. Accordingly, MIC was determined to be 42.5 mg/L with 5-min exposure.

Discussion

As already reported by Mori *et al.*[26], AIT shows strong antifungal activity against aquatic fungi, including *S. parasitica* which is the same strain used in this study. These similar activities were also confirmed. However, there are significant differences between MICs determined by Mori *et al.* and those determined by us. That is, MICs for the mycelial growth is 400 mg/L with 60-min exposure in the former results recorded by Mori *et al.* and 68 mg/L with 60-min in our results, and those for the zoospore germination are 200 mg/L with 60-min and 42.5 mg/L with 5-min exposures, respectively. Here, the only principal difference between the two experimental methods is the incubation temperature of the fungus; 20℃ in the former and 15℃ in the latter, but it is difficult to accredit temperature as the cause of those differences, because there was no statistical difference between fungal growths at the two temperatures as stated above. As another possibility, the cause may relate to the way to prepare the test AIT solution. Because AIT that is oily does not dissolve in water directly, it is necessary in preparation to, first, emulsify the undiluted AIT and then add water to it. However, as Mori *et al.* have failed to mention their method in their report, any further discussion on this matter is impossible.

On the other hand, Isshiki *et al.*[24] have determined MICs for terrestrial fungal growth as follows; 16 mg/L at AIT vapor concentration with 7-day exposure for *Fusarium graminearum*, 22 mg/L for *Penicillium islandicum, P. citrinum, F. oxysporum,* and *Alternaria aleternaria*, 34 mg/L for *F. solani*, 37 mg/L for *Aspergillus niger* and *A. flavus*, and 62 mg/L for *P. chrysogenum* and *Mucor racemosus*. Accordingly, it may be said that MICs of our study are relatively close to these values.

In conclusion, regardless of the cause, MICs in this study are significantly lower than those reported by Mori *et al.* This is favorable in both the issue of safety and of cost for the practical use of AIT as an antifungal agent.

Acknowledgement

We express our sincere thanks to Dr. K. Hatai, Professor of Nippon Veterinary and Animal Science University, Tokyo, and Dr. G. Hoshiai, President of Miyagi Prefecture Inland-Fisheries Research and Development Center, for providing the fungus used in this study.

References

1) WILLOUGHBY, L. G. (1994) Fungi and Fish Disease. Pisces Press, Stirling UK, 57 pp.
2) MEYER, F. P. and JORGENSON, T. A. (1983) Teratological and other effects of malachite green on development of rainbow trout and rabbits. *Trans. Amer. Fish. Soc.*, **112**: 818-824.
3) 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 treatement of eggs. *J. Fish Dis.*, **18**: 239-247.
4) DOERGE, D. R., CHURCHWELL, M. I., GEHRING, T. A., PU, Y. M. and PLAKAS, S. M. (1998) Analysis of malachite green and metabolite in fish using liquid chromatography atmospheric pressure chemical ionization mass spectrometry. *Chem. Res. Toxicol.*, **11**: 1098-1104.
5) BARNES, M. E., SAYLER, W. A. and CORDES, R. J. (2001) Use of formalin treatments during incubation of eyed eggs of brown trout. *North Am. J. Aquacult.*, **63**: 333-337.
6) SCHREIER, T. M., RACH, J. J., and HOWE, G. E. (1996) Efficacy of formalin, hydrogen peroxide, and sodium chloride on fungal-infected rainbow trout eggs. *Aquaculture*, **140**: 323-331.
7) WATERSTRAT, P. R. and MARKING, L. L. (1995) Clinical evaluation of formalin, hydrogen peroxide, and sodium chloride for the treatment of *Saprolegnia parasitica* on fall chinook salmon eggs. *Prog. Fish-Cult.*, **57**: 287-291.
8) MARKING, L. L., RACH, J. J. and SCHREIER, T. M. (1994) Evaluation of antifungal agents for fish culture. *Prog. Fish-Cult.*, **56**: 225-231.
9) BARNES, M. E., EWING, D. E., CORDES, R. J. and YOUNG, G. L. (1998) Observations on hydrogen peroxide control of *Saprolegnia* spp. during rainbow trout egg incubation. *Prog. Fish-Cult.*, **60**: 67-70.
10) KITANCHAROEN, N., YAMAMOTO, A. and HATAI, K. (1997) Fungicidal effect of hydrogen peroxide and malachite green on fungal infection of rainbow trout eggs. *Mycoscience*, **38**: 375-378.
11) KITANCHAROEN, N., YAMAMOTO, A. and HATAI, K. (1998) Effects of sodium chloride, hydrogen peroxide and malachite green on fungal infection in rainbow trout eggs. *Biocon. Sci.*, **3**: 113-115.
12) KITANCHAROEN, N., ONO, A., YAMAMOTO, A. and HATAI, K. (1997) The fungistatic effect of NaCl on rainbow trout eggs saprolegniasis. *Gyobyo Kenkyu (Fish Pathol.)* , **32**: 159-162.
13) KASHIWAGI, M., MAEKAWA, Y., TANAKA, H., YOSHIOKA, M., UENO, R., HOSHIAI, G., HATAI, K., DENO, H. and NAKAMURA, M. (2000) Fungicidal effect of weak alkaline electrolyzed solution on a fish-pathogen Oomycete, *Saprolegnia parasitica. Suisanzoshoku*, **48**: 565-569 (in Japanese with English abstract).
14) KASHIWAGI, M., MAEKAWA, Y., TANAKA, H. and YOSHIOKA, M. (2002) Fungicidal effects of weak alkaline electrolyzed solution on *Saprolengia* infected salmonid eggs. *Suisanzoshoku*, **50**: 363-367 (in Japanese with English abstract).
15) KASHIWAGI, M., OKUMURA, S., NAKANISHI, S., YOSHIOKA, M., UENO, R., HOSHIAI, G. and HATAI, K. (2002) Fungicidal effect of sodium hypochlorite solution on a fish-pathogen Oomycete, *Saprolegnia parasitica. Suisanzoshoku*, **50**: 385-386 (in Japanese with English abstract).

16) KASHIWAGI, M., KHOMVILAI, C., OKADA, A., MAEKAWA, Y., OKUMURA, S., NAKANISHI, S. and YOSHIOKA, M. (2003) Prevention effect of sodium hypochlorite solution on saprolegniasis of salmon eggs. Proceedings of the JSPS-NRCT International Symposium. 15-16 December, Rayong, Thailand. pp. 224-231.

17) KHOMVILAI, C., KASHIWAGI, M. and YOSHIOKA, M. (2005). Fungicidal efficacy of sodium hypochlorite on a fish-pathogen Oomycetes, *Saprolegnia diclina* from Thailand. *Bull. Fac. Bioresources Mie Univ.*, **32**: 39-44.

18) KHOMVILAI, C., KARITA, S., KASHIWAGI, M. and YOSHIOKA, M. (2005) Fungicidal effect of sodium hypochlorite solution on *Saprolegnia* isolated from eggs of chum salmon *Oncorhynchus keta. Fish. Sci.*, **71**: 1188-1190.

19) KHOMVILAI, C., KASHIWAGI, M. and YOSHIOKA, M. (2006) Preventive efficacy of sodium hypochlorite against water mold infection on eggs of chum salmon *Oncorhynchus keta. Fish. Sci.*, **72**: 28-32.

20) KASHIWAGI, M., KISHIHARA, T., WATANABE, T., INABA, T., KATO, T., IKUSHIMA, S., KHOMVILAI, C., and Y OSHIOKA, M. (in press). Alleviation of rainbow trout egg softening caused by sodium hypochlorite as an antifungal agent. *Fish. Sci.*, **72**.

21) NISHIDA, M. (1958) Studies on the pungent component. IV. Antibacterial properties of essential oil of *Eutrema wasabi* Maxim. *Yakugaku Zasshi*, **78**: 435-436 (in Japanese with English abstract).

22) OGAWA, T., MATUZAKI, H. and ISSHIKI, K. (1998) Bacterial control by hydrostatic pressure treatment with addition of allyl isothiocyanate. *Nippon Shokuhin Kagaku Kogaku Kaishi*, **45**: 349-356 (in Japanese with English abstract).

23) HASEGAWA, N., MATSUMOTO, Y., HOSHINO, A., IWASHITA, K. (1999) Comparison of effects of *Wasabia japonica* and allyl isothiocyanate on the growth of four strains of *Vibrio parahaemolyticus* in lean and fatty tuna meat suspensions. *Internat. J. Food Microbiol.*, **49**: 27-34.

24) ISSHIKI, K., TOKUOKA, K., MORI, R. and CHIBA, S. (1992) Preliminary examination of allyl isothiocyanate vapor for food preservation. *Biosci. Biotech. Biochem.*, **56**: 1476-1477.

25) DEPREE, A. J., HOWARD, M. T. and SAVAGE, P. G. (1999) Flavour and pharmaceutical properties of the volatile sulphur compounds of Wasabi (*Wasabia japonica*). *Food Res. Internat.*, **31**: 329-337.

26) MORI, T., HIROSE, H., HANJAVANT, C. and HATAI, K. (2002) Antifungal activities of plant extracts against some aquatic fungi. *Biocont. Sci.*, **7**: 187-191.

27) KITANCHAROEN, N., YUASA, K. and HATAI, K. (1995) Morphological aspects of *Saprolegnia diclina* type isolated from pejerrey, *Odonthedes bonariensis* Type 1. *Mycoscience*, **36**: 365-368.

ミズカビ病原因菌 *Saprolegnia parasitica* に対する西洋ワサビ抽出物の殺菌効果

Chutima KHOMVILAI・柏木　正章・吉岡　　基

三重大学生物資源学部

マラカイトグリーンに代わるミズカビ病防除剤としての西洋ワサビ抽出物の有効性を明らかにするために，その主要成分のイソチオシアン酸アリル（AIT）にミズカビ菌 *Saprolegnia parasitica*（NJM 8604）の菌糸と遊走子を暴露し，これらの発育および発芽阻止効果を検討した。その結果，AIT の菌糸発育阻止最小濃度は 64.8mg/L（60 分間暴露）で，遊走子発芽阻止最小濃度は 42.5mg/L（5 分間暴露）であった。