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In-vitro effects of fungicides on the fungus *Haliphthoros philippinensis*

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Pure cultures of *Haliphthoros philippinensis* isolated from infected *Penaeus monodon* larvae were exposed for 24 hr to varying concentrations of antifungal agents, namely: Benlate, calcium hypochlorite, clotrimazole, copper sulfate, Daconil, formalin, Fungitox, Furanace, griseofulvin, hydrogen peroxide, malachite green, Mystoclin C, phenol, potassium permanganate, Resiguard, Tide, tolnaftate and Treflan. The efficiency of each drug to inhibit the fungus' reproductive process of sporulation as well as its efficiency to prevent mycelial growth were measured. The results establish mycostatic and mycocidal levels of each fungicide.

The inhibitory activities of the 18 test chemicals on the sporulation and mycelial growth of *Haliphthoros philippinensis* after 24 hr exposure are enumerated in Table 1. A "no effect" dose refers to the highest concentration which did not completely stop sporulation. Three types of mycostatic doses have been classified: A, doses that allowed mycelial growth but not motile zoospores; B, doses that significantly inhibited mycelial growth for 24 hr; and C, doses that inhibited mycelial growth for 48 hr. Mycocidal dose refers to the drug concentration that killed the test fungus after 24 hr exposure.

Analysis of the results categorizes these antifungal agents according to levels of mycostatic dose A as the minimum dose for prophylactic treatments. The low mycostatic dose A group with less than 1 ppm effective dose includes calcium hypochlorite, clotrimazole, Furanace and malachite green. The median mycostatic dose A group with effective dose range of 3 to 10 ppm consists of Benlate, Tide and Treflan. The high mycostatic dose group with dose range of 20 to 100 ppm includes Mystoclin C, Fungitox, griseofulvin and tolnaftate. Taking into consideration the economic implication of drug use, the drugs inclusive of the low mycostatic dose A will be promising choices.

Table 3. Mycostatic and mycocidal levels (mg/l) of 18 antifungal agents on *Haliphthoros philippinensis* after 24 hours exposure.

Chemical	Dose Range	"No effect" Dose	Mycostatic A	Mycostatic B	Mycostatic C	Mycocidal Dose
Benlate	10-500	< 10	10-500	500		
Calcium hypochlorite	0.5-2000	< 0.50	0.5-100	20		200
Clotrimazole	0.5-20	0.10(0.05-0.1)	0.5-20	*		
Copper sulphate	0.005-50	> 50		5		
Daconil	1.100	2 (2)	3-100	**		
Formalin	0.5-50	5	6-14	8-12	16-30	40
Fungitox	60-1000	90	100-1000	*		
Furanace	0.001-5	0.1(0.001-0.1)	0.2-0.7	0.8	0.8-1	5
Griseofulvin	100-500	< 100	100-500	100-500		
Hydrogen peroxide	100-500	>500(400-500)				
Malachite green	0.01-10	0.2 (0.1)	0.3-0.4	0.01		0.5
Mysteclin C	10-100	10 (10)	20-100	**		
Phenol	8-1000	8	10-1000	700		
Potassium permanganate	10-100	< 10	10	10		20
Resiguard	0.1-1000	1 (1)	10-100	0.1		200
Tide	0.1-300	9 (0.01-9)	10-80	40	90	100
Tolnaftate	5-500	50 (5-50)	100-500	5	100-500	
Treflan R	0.4-5000	1 (0.4-1)	5-500	0.4	1000	5000

( ) — dose that showed reduced number of zoospores as compared to control
A — dose at which no motile zoospores were detected but allowed mycelial growth.
B — dose which significantly inhibited mycelial growth.
C — dose that inhibited mycelial growth for 48 hours.
* — significant mycelial inhibition concentration was not evaluated due to contaminated controls.
** — aberrant values obtained.

For therapeutic purposes, Furanace, malachite green, formalin and potassium permanganate may be chosen since these retard the growth and kill the pathogenic fungus. Mycocidal doses of Tide, calcium hypochlorite and Resiguard can be utilized for disinfection of discarded runs affected by the pathogen or disinfection of tanks and other larval rearing supplies.

When the test fungus was exposed to Furanace at doses of 0.001 to 0.10 ppm, the number of zoospores detected were relatively less than the control. Absolute inhibition of reproduction was achieved initially at 0.2 ppm. Bacterial contamination however, prevented further evaluation of hyphal effect on this antibiotic. Gross examination of the latter effect showed retardation of mycelial growth at 0.8 to 1 ppm doses. At 5 ppm, the Furanace-exposed fungus died.

The fungitoxic activity of malachite green was demonstrated at a very low level. Its restrictive effect initiated at 0.1 ppm cuts down the number of zoospores released by 50%. Although 0.1 ppm did not effectively stop release of zoospores, hyphal development was significantly affected. Sporulation was entirely prevented at 0.3 ppm while mycelial growth was fatally affected at 0.5 ppm.

In a parallel study on the effect of fungicides on mycolial growth of *Haliphthoros milfordensis* (Abraham and Brown, 1977), malachite green and Furanace showed very low minimum inhibiting concentrations of 0.25 ppm and 2.5 ppm, respectively. This implies a probable affinity in sensitivity of *Haliphthoros* species to said chemicals. Furthermore, both drugs are sensitive to light (van Duijn, 1973; Delves-Broughton, 1974) which will require precautionary measures to maintain prolonged activity of the drugs upon application.

Furanace, because of its antibacterial activity, interferes with nitrification in biological filters, hence, increasing ammonia levels have to be checked. In *Penaeus monodon* larvae, using a 10% active ingredient, Furanace was found to effect a 24 hr LD_{50} (lethal dose) of 1.6 ppm for zoeae and 2 ppm for myses (Gacutan and Llobrera, 1977), indicating the relative toxicity of the present mycostatic levels on *Haliphthoros philippinensis.* It was also reported to have caused morphological damages and reduced swimming as well as feeding functions of the test larvae.

Malachite green has been endorsed for its fungitoxic activity to pathogenic fungi like *Fusarium* at 1.0 ppm for two days (Hatai, Nakajima & Egusa, 1974) and *Lagenidium* sp at 1 ppm, 0.006 to 0.01 ppm and 0.1 ppm, respectively (Armstrong, Buchanan & Caldwell 1976; Bland, Ruch, Salser & Lightner, 1976; Lio-Po, Sanvictores, Baticados & Lavilla, 1982). The tolerance of *P. monodon* to this dye has been established, showing a 24 hr TL5 (tolerance limit) of 0.012 ppm for zoeae and 0.001 ppm for myses and postlarval stages (Lio-Po, Lavilla and Trillo-Llobrera, 1978). This indicates the relative toxicity risks involved if used for prophylactic treatment. Also, despite its therapeutic activity, it has remained unregistered due to its potential carcinogenic and teratogenic properties.

For calcium hypochlorite, impairment of zoospore production was observed at the lowest test dose of 0.5 ppm although mycelial growth was significantly restricted at the 20 ppm dose. Statistical tests showed no variation in mycelial diameter size among doses of 20 to 80 ppm. Death to the test fungus was caused by a 200 ppm treatment.

Mycelial growth inhibition rates for clotrimazole-treated test fungus could not be determined as the untreated fungus get contaminated by bacteria. Evaluation of its effect on reproduction, however, showed a reduction in the population of swimming zoospores at the lowest test dose of 0.05 ppm. Total inhibition of sporulation, however, was obtained at a dose of 0.5 ppm. Examining the colony sizes after 48 hr incubation, there seemed to be no relative variation among all test concentrations.

An "all or none" effect was observed with the formalin-exposed fungal culture. Five ppm did not inhibit spore release but 6 ppm completely restricted this reproductive function. Although inhibition of mycelial growth was detected at the test dose of 7 ppm, the difference from the control was not statistically significant. Inhibition became significant at 8 ppm initially and did not vary until the 12 ppm dose. Prevention of growth at 14 ppm and above proved significantly different from the 8-12 ppm doses. A total kill, however, was realized only at the 40 ppm dose.

Formalin was reported to be effective against the egg fungus (Mawdesley-Thomas, 1972) and *Lagenidium* sp (Lio-Po, et al., 1982) but its registration status has remained indefinite due to its reported neoplastic effect on rats (Shnick and Meyer, 1978). In bioassay experiments with *P. monodon,* it was found to be tolerated by the zoeal, myses and postlarval stages at 5 ppm for a 24-hr exposure.

The lowest dose of 10 ppm potassium permanganate completely prevented sporulation and at the same time significantly reduced mycelial growth by 45%. A 20 ppm dose resulted in death of the test fungus. This chemical is registered for food fish use at 2 ppm (Schnick, Meyer & van Meter, 1979), hence the use of the 10 ppm mycostatic level is met with skepticism. In addition, *P. monodon* exhibited high mortalities when the mycostatic dose was applied (Lio-Po and Lavilla, unpublished data).

With Resiguard, sporulation was only slightly reduced at 1 ppm but completely restricted at 10 ppm. The latter dose, however, kills *P. monodon* larvae upon exposure for 24 hr (Lio-Po and Sanvictores, unpublished data).

Tide caused a slight reduction of zoospores at 0.01 to 0.05 ppm treatments. Total inhibition of sporulation was noted at 10 ppm dose while mycelial growth was significantly inhibited at a higher dose of 40 ppm. A 100 ppm dose caused death of the fungus. This detergent is a cheap, readily available antifungal agent but is not tolerated by *P. monodon* larvae at 20 ppm (Lio-Po and Sanvictores, unpublished data).

Treflan was observed to be fungitoxic to the test fungus *Haliphthoros philippinensis.* Hyphal development was significantly restricted at as low as 0.4 ppm while complete reproductive inhibition was initially exhibited at 5 ppm. This herbicide was established by Armstrong and his co-workers (1976) to have antifungal properties. The present study has proven this to have similar fungitoxic activity towards *Haliphthoros philippinensis.* When tested with *P. monodon* eggs at 10 ppm, it did not significantly affect hatching rate and survival of nauplii after 24 hr exposure. However, older larval stages were sensitive to exposures at similar concentrations (Lio-Po and Sanvictores, unpublished data).

Precautions, therefore, must be observed before actual application of the foregoing recommendations. Preliminary bioassays of larval tolerance to the suggested effective doses should always be made either for prophylactic or therapeutic applications. Other essential factors besides cost and efficacy, such as availability, solubility in freshwater, drug resistance and practical applications should also be considered in choosing the specific chemotherapeutant or its alternatives.

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