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Research Article

Initial Investigations of Cloves and Clove Oil Component as Water Mold Inhibitors

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Abstract

A need exists for less-problematic chemical control of fungal (water mold) infestations on fish eggs during hatchery incubation. This study examined the antifungal properties of cloves *Syzygium aromaticum* and eugenol, the main active ingredient in clove oil. In the first experiment, ground cloves significantly affected the timing of water mold infestation on sesame seeds subjected to fungal spores during static incubation in Petri dishes. Visible fungal growth was observed beginning at 48 hours in control dishes, while growth was not observed in any of the dishes containing ground cloves for the duration of the study. A second experiment used nonviable Chinook salmon *Oncorhynchus tshawytscha* eggs to compare bath and dip 10,000 µg/mL eugenol treatments, with or without ethanol acting as an aqueous mixing agent. There was a significant difference in the timing of fungal growth on the eggs among the treatments. After 288 hours all of the control dishes contained fungal growth. Fungal growth was only observed in one of dishes subjected to a eugenol bath treatment, while the other two replicates were void of any fungal growth during the 504 hour trial. One dish also exhibited fungal growth in the eugenol dip treatment at 384 hours. None of the dishes receiving the eugenol and ethanol bath, or the eugenol and ethanol dip, exhibited any fungal growth. These initial experiments indicate that clove oil, and the eugenol it contains, may have utility in hatchery operations as anti-fungal agents.

Keywords: Clove oil; Eugenol; Water Mold; Fungal Infestation; Fish Eggs

Introduction

Saprolegnia diclina and other zoosporic fungi (water molds) create substantial problems during egg incubation for production fish hatcheries around the world [1, 2]. In the United States, formalin and hydrogen peroxide are the only chemicals approved by the United States Food and Drug Administration for fungal control on incubating salmonid eggs [3]. However, issues with both environmental and human health exist with both of these chemicals [4-6]. Therefore, additional options for fungal control during egg incubation are needed, and several novel therapeutic chemicals have been recently investigated [7-9].

Clove (*Syzygium aromaticum*) oil, and its primary constituent eugenol [10], have been shown to inhibit the growth of a variety of fungi [9-14]. Only two studies have examined possible effects on water molds. A relatively high concentration (500 µg/mL) of eugenol mixed with the solvent dimethyl sulfoxide was required to inhibit *Saprolegnia diclina* growth, with an even higher concentration (1,000 µg/mL) required for fungicidal action [15]. Toxicity to juvenile salmonids at eugenol concentrations of only 63 µg/mL for 10 min has also reported [15]. A more recent experiment indicated that, based on in-vitro experiments, the concentrations of clove oil required for fungal control led to pre-eyed rainbow trout *Oncorhynchus mykiss* egg mortality [16]. However, they noted

no antifungal effects during actual egg incubation, but the clove oil treatments were only administered at most three times per week. In contrast, antifungal treatments on incubating eggs in production fish hatcheries are typically applied daily [17].

There have been no published studies investigating the daily use of eugenol as an anti fungal on fish eggs. In addition, no studies have reported if ground cloves themselves, without the isolation of eugenol, are able to control water molds. The objective of this study was to first evaluate the effectiveness of ground cloves as a fungicide in fish hatchery water known to produce water mold infestations and then subsequently determine if clove oil would be an effective fungicide in a simulated production aquaculture egg treatment scenario.

Materials and Methods

All experiments were conducted at McNenny State Fish Hatchery, Spearfish, South Dakota, using well water (total hardness as $CaCO_3$, 360 mg/L; alkalinity as $CaCO_3$, 210 mg/L; pH, 7.6; total dissolved solids, 390 mg/L) known to contain *Saprolegnia diclina* zoospores [18].

Experiment 1.

Sesame *Sesamum indicum* seeds were used as a surrogate for fish eggs to determine the timing of fungal colonization [19, 20]. Prior to use, the seeds were disinfected and seed coats broken by boiling for a minimum of 20 minutes [21]. Three sesame seeds each were added to eight sterile 9.5-cm plastic Petri dishes. Each dish contained 28 mL of hatchery well water. Four of the dishes received 0.5 g of ground cloves *Syzygium aromaticum* (Frontier Natural Products Co-op, Norway, Iowa, USA), while the remaining four control dishes contained only sesames seeds and water. The dishes were incubated at 18° C and observed daily for fungal growth. Observations of fungal colonization were made daily, with the presence of hyphae attached to a seed used to indicate colonization.

Experiment 2.

Nonviable Chinook salmon *Oncorhynchus tshawytscha* eggs were used in this experiment instead of sesame seeds. Three eggs were placed in each Petri dish. Three control dishes contained only salmon eggs and 28 mL of hatchery well water. Three experimental solutions were used in this experiment. The first treatment used 28 mL of a 10,000 µg/mL eugenol solution in three dishes. Eugenol, a clove oil extract (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was mixed with hatchery well water. Eugenol was used in this experiment, rather than the ground cloves used in Experiment 1, to allow for a comparison to other published studies. The second treatment also used 28 mL of a 10,000 µg/mL eugenol solution, but in addition contained 50,000 µg/mL ethanol to facilitate the mixing of the eugenol in the aqueous solution [22, 23]. The final treatment was a 50,000 µg/mL ethanol solution with hatchery water which did not contain any eugenol. Each experimental solution was placed into three Petri dishes along with three salmon eggs as a prolonged bath treatment. In addition, each of the experimental solutions was used in 15-minute dip treatments, in an attempt to mimic typical salmonid egg treatment protocols during hatchery production [24]. These dip treatments were performed by removing the salmon eggs from a Petri dish containing just water, placing the eggs in another dish containing the experimental solution for 15 minutes, rinsing the eggs in hatchery water after the 15 minute treatment, and returning the eggs to the Petri dishes containing just hatchery water. Three dishes were used for each solution experiencing the dip treatment. Thus, 21 Petri dishes were used in this experiment, with the three solutions used in both bath and dip treatments (N=3, with one control and six treatment groups). The dishes were maintained at 18o C and checked daily for fungal growth for 21 days.

Statistical Analysis

Data were analyzed in Experiment 1 using the Mann-Whitney non-parametric test. The Kruskal Wallis one-way analysis of variance by ranks was used to analyze data in Experiment 2, with pair-wise mean comparisons performed using the Conover-Inman Test. Significance was pre determined at $P < 0.10$, which is acceptable for exploratory or introductory studies [25, 26]. All statistical analysis was conducted using the SPSS (9.0) statistical analysis program.

Results

Experiment 1.

The timing of fungal growth in the control dishes was significantly different ($P = 0.046$) than in the dishes containing ground cloves. Fungal growth was evident in two of the control dishes at 48 hours. At 96 hours fungal growth was observed in an additional control dish, while the remaining control dish did not exhibit any fungal growth. No fungal growth was observed in any of dishes containing ground cloves at any time during the trial.

Experiment 2.

There was a significant difference ($P=0.067$) in the timing of fungal growth among the treatments. After 288 hours, all of the control Petri dishes contained fungal growth (Table 1). Fungal growth was observed in only one of dishes subjected to a eugenol bath treatment, while the other two replicates were void of any fungal growth during the 504 hour trial. One dish also exhibited fungal growth in the eugenol dip treatment at 384 hours. Fungal growth was evident at 288 and 384 hours in two of the dishes treated with the ethanol dip. None of the dishes receiving the ethanol bath, eugenol and ethanol bath, or

the eugenol and ethanol dip exhibited any fungal growth. Pair-wise comparisons indicated that the timing of fungal growth was significantly different in the control treatment compared to all of the other treatments except the ethanol wash.

Treatment	Dish (replicate)	Initial Fungal Growth (hour)
Control (no chemicals)[z]	1	216
	2	216
	3	288
Eugenol bath[yx]	1	192
	2	no growth
	3	no growth
Ethanol bath[x]	1	no growth
	2	no growth
	3	no growth
Eugenol + ethanol bath[x]	1	no growth
	2	no growth
	3	no growth
Eugenol dip[yx]	1	384
	2	no growth
	3	no growth
Ethanol dip[zy]	1	288
	2	384
	3	no growth
Eugenol + ethanol dip[x]	1	no growth
	2	no growth
	3	no growth

Table 1. Timing of the first observation of fungal growth in Petri dishes containing nonviable Chinook salmon eggs subjected to antifungal treatments of eugenol or ethanol during a 504 hour trial. Different superscript letters indicate significant differences ($P < 0.10$; N=3).

The ethanol wash results in turn were significantly different from all of the other treatments except the control, eugenol bath, and eugenol wash.

Discussion

Similar to this study, other researchers have also reported anti-fungal properties of clove oil and eugenol, but at different concentrations and treatment times. *Saprolegnia* spp. growth was inhibited on rainbow trout eggs with a 10 min application of 250 µg/mL of eugenol [15]. Fungal control was also observed with clove oil at 10,000 mg/L [16]. The results from these experiments and the present study show the promise of eugenol as a fungicide during hatchery egg incubation. Typical hatchery anti-fungal incubated egg treatments occur daily for fifteen minutes [17]. The success of the fifteen minute dip treatment in the second experiment of the current study shows the potential of easy integration into existing hatchery treatment procedures, ideally replacing formalin or hydrogen peroxide.

This study did not evaluate any potential detrimental effects of cloves or eugenol on egg mortality. Near-total mortality of rainbow trout eggs treated with a solution of 1,000 mg/L of clove oil and ethanol has been reported [16]. These results are difficult to interpret. Clove oil contains numerous impurities. Further, eugenol is only one of several active ingredients in clove oil, which also include eugenyl acetate, caryophyllene, 2-heptanone, ethyl hexanoate, humulenol, a-humulene, calacorene, and calamenene [27]. Additionally, the study indicating clove oil-induced mortality [16] treated the eggs either once or three times a week until hatch. Due to the treatment timeframe, it is possible that there is a sensitive period during egg development which caused the high mortality. Although an effective egg fungicide, hydrogen peroxide is particularly toxic during blastopore closure [28]. In addition, in the experiment indicating clove oil-induced mortality [16], the eggs were exposed to clove oil after the eyed-egg stage of development, when eggs can be safely handled and anti-fungal chemicals do not necessarily need to be applied [29]. Because of the questions regarding methodology, and the differences in clove oil and eugenol, further experimentation is needed regarding mortality.

The fungal growth at 192 hours in one of the eugenol bath treatment dishes was unexpected. While this outlier still retarded fungal growth better than the controls, it was very different than the other replicates. It is possible that one or more of the eggs in this dish could have been infested with non-visible growth that was only retarded by the eugenol bath treatment. Also, the salmon eggs used in the experiment were not sterilized prior to inclusion in the study and came directly from hatchery water containing abundant fungal spores [18].

The ethanol results observed in this study are similar to those

reported in the literature [16]. The continuous bath of 50,000 µg/mL ethanol had no growth, similar to the results reported for a 70,000 µg/mL continuous bath [16]. In contrast, the current study showed growth in two of three dishes receiving daily 15 min, 50,000 µg/mL ethanol treatments, while growth has been reported in all of the replicates subjected to a 15 min treatment of the 70,000 µg/mL ethanol only once or three times per week [16]. Differences in water chemistry [30] or *Saprolegnia* strains may be responsible for the different results between the two studies. The strain prevalent in the water we used grows prolifically on dead eggs, necessitating daily anti-fungal treatments to avoid near-total egg mortality during egg incubation [31].

In conclusion, cloves and eugenol effectively controlled the growth of fungus during this study which simulated the typical procedures used during egg incubation. However, further research is needed to further refine the effectiveness of eugenol treatment concentrations on fungal control. In addition, the potential toxic effects of different eugenol concentrations during the stages of egg development should also be investigated.

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