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Bioprophylaxis of Saprolegniasis in Incubated *Clarias gariepinus* Eggs Using Pyocyanin Extracted from *Pseudomonas aeruginosa*

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***Abstract*—**Saprolegniasis is a major pathogenic infection that contributes significantly to poor hatching rates in incubated fish eggs in the African catfish hatchery in Nigeria. Malachite green, known to be very effective against this condition, has been banned because it is carcinogenic. There is therefore the need for other effective, yet safer method of controlling saprolegniasis in incubated fish eggs. A total of 50 ml crude, chloroform extract of pyocyanin from which solvent was removed to attain 30 ml, having a concentration of 12.16 ug/ml was produced from 700 ml broth culture of *Pseudomonas aeruginosa* isolated from a previous study. *In vitro* susceptibility of the fungus was investigated by exposing fungal infected eggs to two different time-concentration ratios of pyocyanin; 0.275 ug/ml and 2.75 ug/ml for 1 and 24 h, and 5 mg/L malachite green as positive control while normal saline was the control. Efficacy of pyocyanin was evaluated using the degree of mycelial growth inhibition in the different treatments. Fertilized *Clarias gariepinus* eggs (between 45 to 64 eggs) were then incubated in 20 ml of medium containing the similar concentrations of pyocyanin and malachite green, with freshwater as control for 24 hours. Hatching rates of the incubated eggs were observed. Three samples of un-hatched eggs were taken from each medium and observed for the presence of fungal pathogens using microscopy. Another batch of three samples of un-hatched eggs from each treatment was also inoculated on Sabourand dextrose agar (SDA) using Egg-Agar Transfer technique to observe for fungal growth. Mycelial growth was inhibited in fungal infected eggs treated with 2.75 ug/ml for 24 h and the 5 mg/L malachite green for both 1 h and 24 h. The mortality rate was 100% in fertilized *C. gariepinus* eggs exposed for 24 h to 0.275 and 2.75 ug/ml of pyocyanin. The mortality rate was least in the malachite green followed by the control treatment. Embryonic development was observed to be arrested in the eggs treated with the two pyocyanin concentrations as they maintain their color but showed no development beyond the gastrula stage, whereas viable eggs in the control and malachite green treatments developed fully into healthy hatchlings. Furthermore, microscopy of the un-hatched eggs revealed the presence of a protozoan ciliate; *Colpidium* sp. (Tetrahymenidae), as well as a pathogenic fungus; *Saprolegnia* sp. in the control, but not in the malachite green and pyocyanin treatments. Growth of *Saprolegnia* sp. was also observed in SDA culture of un-hatched eggs from the control, but not from pyocyanin and malachite green treated eggs. Pyocyanin treatment of incubated eggs of *Clarias gariepinus* effectively prevented fungal infection in the eggs, but also arrested the development of the embryo. Therefore, crude chloroform extract of pyocyanin from *Pseudomonas aeruginosa* cannot be used in the control of Saprolegniasis in incubated *Clarias gariepinus* eggs at the concentration and duration tested in this study.

***Keywords*—**African catfish, bioprophylaxis, embryo, saprolegniasis.

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I. Introduction

COMMERCIAL production of the African catfish in Nigeria has developed into a vibrant industry, contributing significantly to the country's GDP. The industry is private sector driven and huge resources have been committed to fish production by culture making Nigeria the leading producer of catfish in Africa [1], [2]. However, hatchery propagation of catfish seeds faces crippling challenges that include incidence of diseases, among which is the endemic Saprolegniasis [3], [4]. Controlling this fungal disease condition has become even more challenging especially with the ban placed on malachite green, an effective fungicidal agent, because it is carcinogenic [5], [6]. There is therefore the need for another effective but safer control measure against Saprolegniasis in incubated fish eggs.

This study was therefore designed to investigate the efficacy of Pyocyanin, a phenacin derivative extracted from *Pseudomonas aeruginosa* in the control of Saprolegniasis in incubated eggs of C*larias gariepinus*. According to [7] and [8], pyocyanin possesses antifungal activity expressed in the strong antagonism against *Candida albicans* and *Aspergillus fumigatus*, and could therefore be effective against *Saprolegnia* sp. associated with mortality in incubated catfish eggs.

II. Materials and Methods

Resuscitation of Pseudomonas aeruginosa and Preparation of Broth Culture

Pseudomonas aeruginosa isolated in an earlier study (unpublished), and stored in refrigerator at 4 °C was resuscitated in nutrient agar, following the standard procedure of agar preparation and sterilization by autoclave at 121 °C for 15 minutes at 1 Atm pressure. A 700 ml broth culture of pure isolate was then prepared following the same protocol.

Extraction of Pyocynin from Broth Culture

Pyocyanin was extracted from *Pseudomonas aeruginosa*, using chloroform at 50% of the broth culture as earlier described [9]. The absorbance of the aqueous fraction was then measured at 520 nm wavelength. The optical density of 0.032 obtained was multiplied by a factor (17.072) and a dilution factor of 5 to determine pyocyanin concentration [9].

Isolation and Identification of Fungal Pathogen

Purposive sampling method was used to collect few fungal infected, dead, incubated C*larias gariepinus* eggs from two

major catfish hatcheries in Ibadan, Nigeria during three different controlled spawning exercises. Mycelia samples collected from the infected eggs that have turned whitish with fluffy, cottony growth around them were stained with lactophenol cotton blue, and observed under the microscope (x200 and x400). Furthermore, similar mycelia samples were aseptically inoculated unto Sabouraud Dextrose Agar (SDA) made selective with the incorporation of 0.05 mg/L Chloramphenicol and 0.4 mg/ml Actidion, incubated at 25 °C for 48 hours [4]. Inoculum from fungal growth from SDA culture were similarly stained and also observed under the microscope. Fungal identification was based on hyphae and organ morphology.

In vitro Pyocyanin Susceptibility Test

A batch of 10 dead catfish eggs with fungal growth were disinfected in two different concentrations (0.275 µg/ml and 2.75 µg/ml) of pyocyanin and two other batches of same dead eggs were introduced into freshwater (negative control) and 5 mg/L malachite green (positive controls) for 1 h and 24 h, respectively. Three of the fungal infected eggs disinfected in each of the different concentrations of pyocyanin, freshwater and malachite green were then aseptically transferred onto SDA in Agar-Egg Transfer technique, to observe for fungistatic or fungicidal action of pyocyanin and malachite green.

Egg Disinfection Trial

Between 45 and 64 fertilized eggs of *Clarias gariepinus* produced by controlled spawning were disinfected in pyocyanin. The pyocyanin concentrations used were those to which *Saprolegnia* spp. were susceptible in the *in vitro* trial, for the corresponding period of exposure. The same was done for 5 mg/L malachite green for 1 hr, and fresh water. The hatching rate was then determined for the different experimental treatments.

III. Results

The resuscitated *Pseudomonas aeruginosa* grew in the characteristic bluish green, flat colonies that contained Gram-negative, slender rods which are motile. The organism is oxidase and catalase positive, indole, methyl red and Voges Proskauer (VP) negative, reduces nitrate but did not produce hydrogen sulfide. Microbact™ 24E, Gram-negative Identification System (MGIS) for Gram-negative bacilli was used for identification.

A total of 50 ml pyocyanin having a concentration of 2.75 ug/ml was extracted from 700 ml broth culture of *P. aeruginosa*. The bluish extract became red when acidified with 0.1 N hydrochloric acid. The solvent was removed using rotary evaporator, and solution reconstituted with Phosphate buffered saline (PBS) to attain 30 ml, having a concentration of 12.16 ug/ml stock solution.

Culture of inoculum from dead fish eggs yielded white cottony growth on SDA and three fungal species of the family Saprolegniaceae namely: *Saprolegnia* sp., *Leptolegnia* sp. and *Aphanomyces* sp. were identified in the incidence of saprolegniasis in incubated *Clarias gariepinus* eggs in the catfish hatcheries surveyed in this study. Identification was based on morphological features of the fungi. Branched, septate hyphae filled with spores, characteristic of *Leptolegnia* sp. were observed (Fig. 1), while *Saprolegnia* sp. featured large, unbranched and non-septate hyphae with ova-containing oogonium. Unbranched, nonseptate hyphae with terminal, spore filled sporangium also observed are characteristic of *Aphanomyces* sp. (Fig. 2).

Fig. 1 *Leptolegnia* sp. with spore-filled, branched, septate hyphae

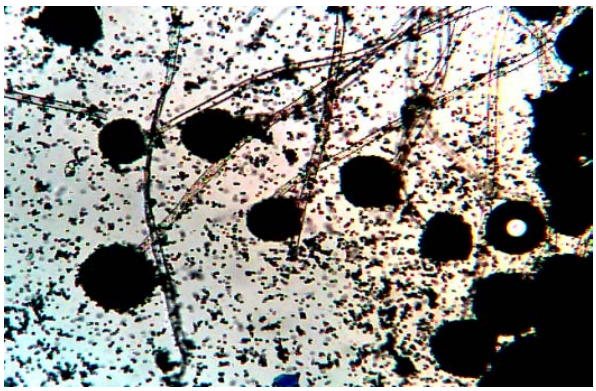

Fig. 2 *Aphanomyces* sp. with unbranched, nonseptate hyphae having terminal, spore-filled sporangium

TABLE I
Intensity of Mycelia Growth on SDA Following Disinfection of Fungal-Infected Eggs

	Duration of Exposure (Hr.)	Intensity of Mycelia growth
0 µg/ml Pyocyanin (Freshwater)	1	+++
	24	+++
0.275 µg/ml pyocyanin	1	+++
	24	+
2.75 µg/ml pyocyanin	1	+
	24	-
5 mg/L Malachite Green	1	-
	24	-

+++ Massive growth, + Low growth, - No growth

As shown in Table I, there was no fungal growth when dead, infected *Clarias gariepinus* eggs disinfected in 2.75

µg/ml for 24 h and malachite green for both 1 h and 24 h were inoculated on SDA. However, at a concentration of 2.75 µg/ml pyocyanin for 1 h and 0.275 µg/ml for 24 h treated eggs showed very little mycelial growth. Furthermore, there was massive mycelial growth on SDA when eggs treated with freshwater and 0.275 µg/ml pyocyanin for 1 h were transferred onto the culture medium.

As shown in Table II, mortality rate was 100% in fertilized *C. gariepinus* eggs exposed for 24 h to 0.275 and 2.75 ug/ml of pyocyanin. The mortality rate was least in malachite green followed by the control treatment. Embryonic development was observed to be arrested in the eggs treated with the two pyocyanin concentrations as they maintain their color (Fig. 3) but showed no development beyond the gastrula stage (Fig. 4), whereas viable eggs in the control and malachite green treatments developed fully into healthy hatchlings (Fig. 5). Furthermore, microscopy of the un-hatched eggs revealed the presence of *Saprolegnia* sp. in the control but not in the malachite green and pyocyanin treatments. Growth of *Saprolegnia* sp. was also observed in SDA culture of un-hatched eggs from the control, but not from pyocyanin and malachite green treated eggs. The observation of a protozoan ciliate; *Colpidium* sp., (Tetrahymenidae), in the control but not in the malachite green and pyocyanin treatments was an accidental finding (Fig. 6).

TABLE II
HATCHING RATES OF FERTILIZED EGGS IN THE DIFFERENT DISINFECTION TREATMENTS

	Control (Freshwater)		Malachite green 5mg/L		Pyocyanin 0.275 ug/ml		Pyocyanin 2.75 ug/ml	
Replicates	1	2	1	2	1	2	1	2
Hatched Eggs	7	16	17	14	0	0	0	0
Unhatched Eggs	41	48	28	41	54	54	58	56
Total No. of eggs	48	64	45	55	54	54	58	56
Hatching rates (%)	14.6	22.2	37.8	37.8	0	0	0	0

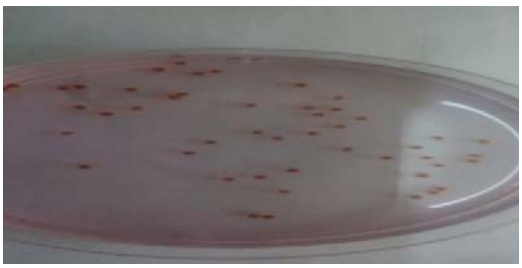

Fig. 3 Arrested Embryonic Development in Eggs Treated with Pyocyanin

IV. DISCUSSION

Catfish production has transformed into an industry in recent years contributing significantly to the GDP of Nigeria [1], [11]; thus, the investment committed to the industry should be protected. Inadequacy in catfish seed supply constituting a major constraint in the industry is due to many factors including fungal infection of incubated eggs that causes poor hatching rates. The three species of the family Saprolegniaceae observed in this study, namely *Saprolegnia* sp., *Leptolegnia* sp. and *Aphanomyces* sp. are among those earlier observed by other scientists [6], [10], and showed that multiple fungal pathogens are involved in the infection of incubated fish eggs.

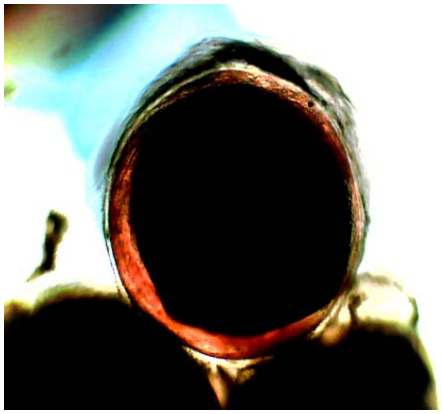

Fig. 4 Arrested Embryonic Development in Eggs Disinfected with Pyocyanin (x40)

Fig. 5 Normal Hatchling Observed in the Malachite Green and Freshwater Treatment (x40)

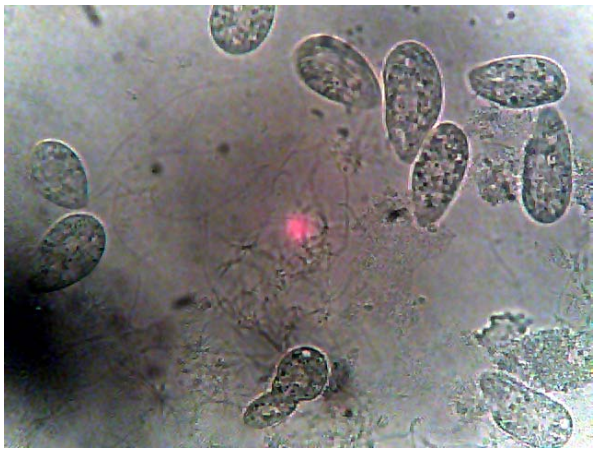

Fig. 6 *Colpidium* sp. Observed in Freshwater Treatment by Microscopy (x400)

Pyocyanin produced by *P. aeruginosa* have long been

recognized as a biological control agent against fungal and bacterial organisms, as well as protozoan parasites [8], [12], [13]. However, the two concentrations of pyocyanin used in this study: 0.275 ug/ml and 2.75 ug/ml, were observed to arrest embryonic development in incubated catfish eggs at the gastrula stage, hence are not suitable for the disinfection of incubated catfish eggs against Saprolegnia infection. This observation may be due to the toxic effect of pyocyanin mediated hydrogen peroxide production that is known to cause several defects in cells, such as detachment of cells from their substratum, shrinkage, blebbing of cell membranes, and apoptosis that leads to cell death [14]. This explains the arrested development of embryo in pyocyanin treated incubated eggs, and the 0% hatching rates observed thereafter. *Saprolegnia* sp. was re-isolated on SDA from dead eggs under the control treatment but not in any of the pyocyanin treatments. This lent credence to antifungal efficacy of pyocyanin even in comparison with malachite green known for high antifungal efficacy.

V. Conclusion

Three fungal species of the family Saprolegniaceae; *Saprolegnia* sp., *Leptolegnia* sp. and *Aphanomyces* sp. were identified in saprolegniasis cases observed in incubated *Clarias gariepinus* eggs, in the catfish hatcheries surveyed in this study. All species of fungal pathogens isolated were also observed to be highly susceptible to pyocyanin disinfection at the 2.75 ug/ml and 24 hours duration of exposure only. However, *in vivo* susceptibility trials revealed that pyocyanin is toxic to developing embryo of *Clarias gariepinus* since the development was arrested at the gastrula stage leading to eventual death of all incubated eggs. Therefore, though pyocyanin was observed to be a potent antifungal and possibly antiprotozoan agent, it is not suitable for the control of saprolegniasis in incubated eggs of *Clarias gariepinus* at the concentration and duration of exposure used in this study.

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