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Studies on the identification and control of pathogen *Saprolegnia* in selected Indian major carp fingerlings at mid hill altitude

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Abstract

The Indian major carp cultured in ponds in the North Eastern hilly states of India frequently suffer from fungal disease during winter months resulting in mass mortality. This study examined the pathogenic fungi isolated from farmed raised Indian major carp fingerlings and identified as *Saprolegnia*. For treatment, the diseased fish were exposed to 4g salt per litre of water for 2 min followed by dip treatment with 5ppm $KMnO_4$ for 10 min, thrice every week for a period of 6 weeks. The treatment resulted in recovery from the disease after 6 weeks from the beginning of treatment. Soon after recovery, the pond management practices such as removal of pond bottom soil, application of lime and replenishment with freshwater were followed in the infected ponds. Our study concluded that rapid decrease in pond water temperature from 22 to 8°C that remains low for months together coupled with increased water pH (9) and decreas dissolved oxygen (4ppm) causes saprolegniasis to the fingerlings of Indian major carps.

Key words

Saprolegnia, Pathogenic fungi, Major carp

Introduction

The Indian major carps *(Catla catla, Labeo rohita and Chirrinus mrigal),* minor carps *(Labeo bata, Labeo gonius)* and exotic carps *(Ctenopharygodon idella, Hypothalmicthis molitrix and Cyprinus carpio)* are the major fish species cultured in earthen ponds in the region (Vinod *et al.*, 2004). In many parts of the north eastern hill region, the cultured fishes frequently suffer from fungal disease, especially during the months from late November to early February when water temperature falls below 10°C (Naskar *et al.*, 2005). The disease causes mass mortalities and major financial losses to the poor fish farming community. Some of the typical signs of disease are skin lesions associated with a 'dry' mucus-depleted skin, erratic swimming behavior and sunken eyes (Majhi *et al.*, 2005).

Indeed, every freshwater fish is exposed to at least one species of fungus during its lifetime (Neish, 1997 and Noga, 1996), starting from the embryonic stage through adulthood (Bruno and Wood, 1994). Several authors have reported occurrence of fungal diseases in many commercially important fish species such as Channel catfish cultured in earthen ponds in the southern

U.S. (Bly *et al.*, 1992); young and adult Salmonids cultured in northern coastal waters of Japan (Hatai and Hoshiai, 1992); Pike cultured in Scotland (Willoughby, 1985); Bass reared in cages in U.S. (Noga, 1996), mass-scale culture of Tilapia during winter months in Egypt (Zaki *et al.*, 2008), Roach, Carp, Lamprey, Sturgeon, Barramundi, Mullet, Kissing gourami, Guppy, Swordfish and Platyfish (Bruno and Wood, 1994).

Recently, we observed a very similar diseased condition in our fish farm at ICAR complex for north eastern hill region, Barapani, Meghalaya, during the winter months, causing severe mortality of Indian major carp *(Catla catla, Labeo rohita and Chirrinus mrigal)* fingerlings. In the present study, we examined the key pathogen associated with the disease and recorded the developmental phases of infection (from the first sign of infection to the death), pertaining to the conditions of mid hill altitude.

Materials and Methods

The north eastern hill (NEH) region of India lying between 21.5° to 29.5° N latitude and 85.5° to 97.5° E longitude are characterized by difficult hilly terrains, which range from a few 100 feet to over 28,000 feet a msl . The climate of the region also varies from tropical through sub-tropical to temperate types. The rainfall

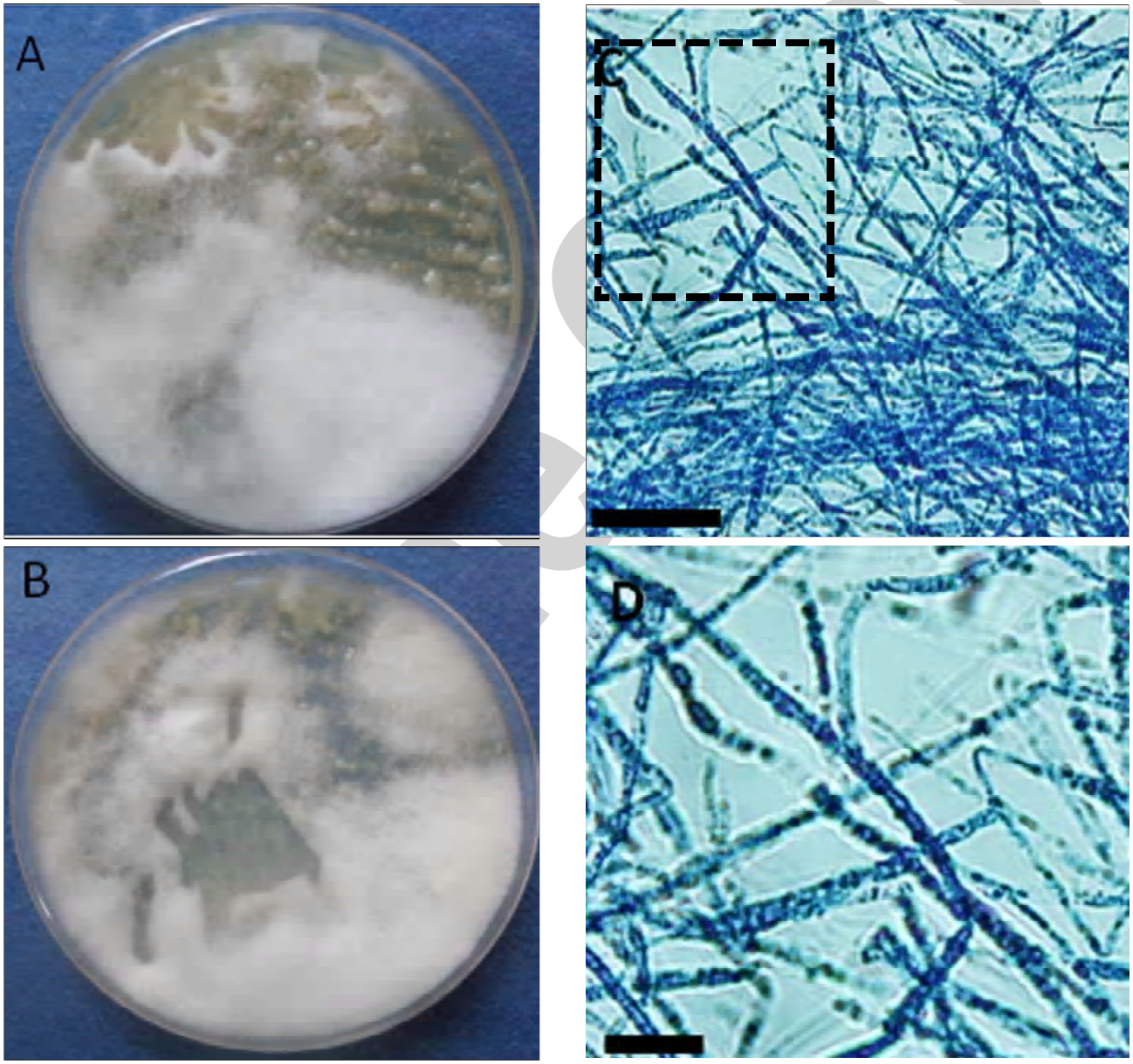

Fig. 1: Growth and morphological characterization of isolates. View of a dextrose agar plate on 5 days (A) and 10 days (B) from the beginning of incubation at 22°C. C-D: Morphological characterization under light microscope shows zoospores of *Saprolegnia* (B is the higher magnification of box shown in A). Scale bar indicates 200µm (C) and 50µm (D).

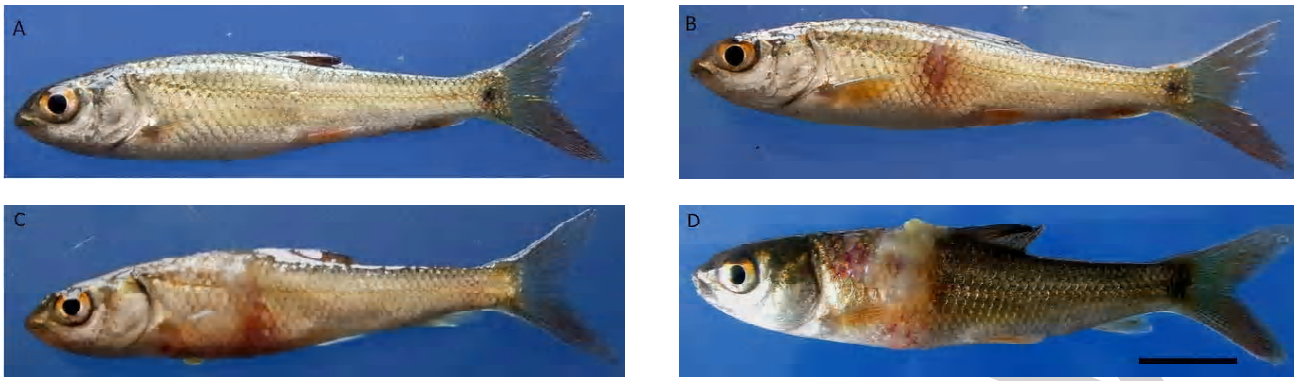

Fig. 2: Steps of *Saprolegnia* infection in IMC (*Catla catla, Labeo rohita* and *Chirrinus mrigal*) fingerlings. (A) A healthy fingerling showing intact body morphology, (B) The first sign of infection. Note appearance of red patches of filamentous mycelium associated with skin lesion (arrow), (C) In the second step, the mucus layer over the skin of the fish turns semi-dry, gradual spread of red patch and appearance of cotton-like structure (arrow) and, (D) Rampant of "cotton-wool" like structure that radiates out in a circular pattern (arrow). Scale bar indicate 1cm.

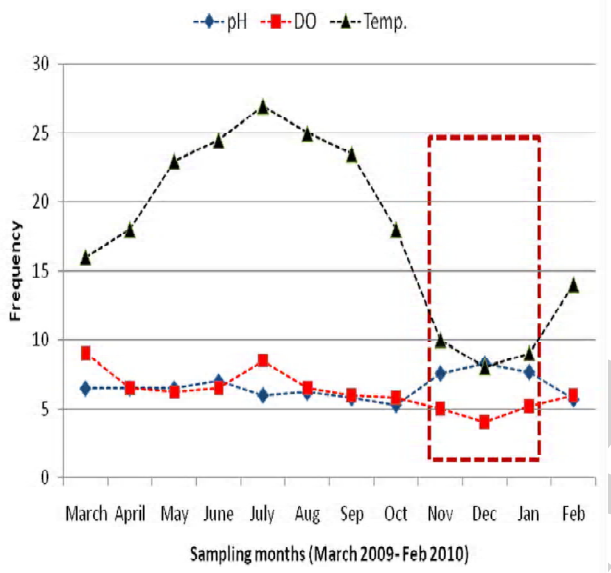

Fig. 3: Water quality parameters recorded during March 2009 to February 2010 in the *Saprolegnia* infected pond. Box inset on the figure represent the critical period for *Saprolegnia* infection.

varies from 1100 to 12,500mm. Similarly a maximum difference of about 7 to 12°C in average minimum temperature occurs at high and mid altitude locations during the winter months (Majhi *et al.*, 2006).

Fish maintenance and water quality parameters: The nursery ponds of size 0.04 ha (n=5) are located at 22.5° N latitude and 88.5° E longitude. The average water depths of the ponds were 1.5 m. The Indian major carp fingerlings *(Catla catla, Labeo rohita and Chirrinus mrigal)* of 4-5 months old (mean body weight 5.2 g and mean body length 6.8cm) produced in our hatchery facility were stocked in nursery ponds at the rate of 8 nos sq mt^{-1}. The fishes were regularly fed with supplemental feeds (rice polish and mustard oil cake) at 5% body weight. The water quality parameters (pH and dissolved oxygen) and air and water temperature were recorded weekly and analyzed using standard methods (APHA., 2005).

Microbiological assay: Ten each live infected and control fish were randomly collected, maintained in aseptic conditions, from each ponds after 2, 6, 10 and 20 weeks from the beginning of infection. The fishes were sacrificed and body weight recorded. The cotton-wool appeared on the body surface of fishes were removed by sterile inoculating loop and incubated in Sabauravd's dextrose agar (HiMedia, Mumbai, India) plates (pH 5.6) and stored at 22-30°C for 5-10 days. The plates were observed everyday at 10:00 hrs for growth. For identification of fungus, the cultures were subjected to lactophenol cotton blue (LCB) stain (HiMedia, Mumbai, India) following the protocol of Chakraborti (2003), Thomas *et al.* (1991), Pelczar *et al.* (2008). Briefly, a drop of LCB stain was placed on the centre of slide. By using sterilized mounting needle, a small portion of culture was mixed gently with the LCB stain. A cover slip was placed gently to avoid formation of air bubble and was examined under a light microscope at magnifications between 10-100X and the image were captured using a digital camera.

Disease treatments: The fishes at the initial stage of infection were caught alive and grouped into two (n=30). The fishes in each group were subjected to treatments (A: dip treatment with 4 g salt per litre of water for 2 min.; B: dip treatment with 4g salt l^{-1} of water for 2 min followed by 5ppm $KMnO_4$ for 10 min). The treatments were given thrice every week at 10:00 hrs in a plastic tub (capacity: 50 l), up to 6 weeks from the beginning. The observations were recorded every day for recovery and/or improvement in condition, growth attainment and mortality caused in each treatment.

Statistical analysis: Measured parameters for fish mortality during treatment period between 0-20 weeks, growth attainment and improvement in disease condition following treatments were compared by one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison test. Data are presented as mean and differences between groups were considered statistically significant at $P<0.05$.

Results and Discussion

The microscopic observations of cultures suggest that the pathogen belongs to genus *Saprolegnia* (Fig. 1a-b) and presumably were secondary product of asexual zoospore (Fig. 1c-d). In general, the secondary zoospores are more motile for a longer period of time than primary zoospores (Shah, 2010) and are the main dispersion phases of *Saprolegnia* (Pickering and Willoughby., 1982). Additionally, secondary zoospores are also considered the infectious spore of *Saprolegnia* (Bruno and Wood, 1994; Hatai and Hoshiai, 1992) that causes fish mortality.

Saprolegnia is ubiquitous in freshwater ecosystems and is the main genus of water molds responsible for significant fungal infections of freshwater fish and eggs (Pelczar *et al*., 2008). The infection of fish with *Saprolegnia* is generally termed "saprolegniasis" (Roberts, 1989). In the present study, we recorded severe mortality of fingerlings by saprolegniasis during the winter months i.e. early November 2009 to late January 2010. We observed that from the onset of infection fishes died within 12-15 days (Fig. 2 a-d). The first sign of infection were visible red or gray patches of filamentous mycelium (Fig. 2b). The development of patches was probably due to rubbing of body surface to a hard substratum following attachment of filamentous mycellium that causes itching. Subsequently, during acute infection that takes 7-8 days from beginning of infection, appearance of cotton-like structure (Fig. 2c) that radiate out in a circular, crescent-shaped or whorled pattern (Fig. 2d) and from then the fish died within 3-4 days. Several authors have reported that *Saprolegnia* invades epidermal tissues, generally beginning on the head or fins and spreads over the entire surface of the body (Zaki, 2008; Neish, 1997; Willoughby and Roberts, 1992). Contrary, in the present investigation we observed that the red patches first appeared in the mid part of the body surface and then gradually spread to other parts. This allowed us to speculate that the site of infection and also the patterns might vary between farm raised fish, as in the present case, and wild fish (Beakes, 1982; Pickering and Willoughby, 1982).

The physiological state of the fish and the environmental conditions generally determines if a fungal infection will be successfully established (Neish, 1997). In the present investigation, a sudden decrease in water temperature (from 20°C to <8°C) that remained low for longer duration coupled with increase in water pH (>9) caused saprolegniasis in cultured fish (Fig. 3). Bruno and Wood, (1994) reported that sudden changes in temperature can make fish vulnerable to saprolegniasis, due to the increased physiological stress. This may be particularly true in the present case due to the fact that the Indian major carp species grow better in a temperature range of 26-33°C (Das *et al*., 2004) and their physiology such as feeding, swimming, oxygen consumption and thermal regulation rates are influenced significantly in low-temperature condition. Generally, *Saprolegnia* has a fairly wide range of temperature tolerance from 3 to 33° C (Aly and El Ashram, 2000; Willoughby and Roberts, 1992). However, they attack fishes that have been stressed or have weak immune system (Bruno and Wood, 1994; Pickering, 1994). Neish (1997) suggested that immunosupression driven by environmental parameters provide a mechanism that causes the transformation of normally non-pathogenic organisms, including *Saprolegnia*, to become pathogenic.

Generally, fungal infections are difficult to treat, especially during acute condition. Nevertheless, there are few chemicals approved for use in aquaculture (Fitzpatrick *et al*., 1995; Meyer, 1991) for treatment of such diseases. Malachite green is considered the most effective chemical for controlling *Saprolegnia* (Willoughby and Roberts, 1992). However, because of concerns about its potential teratogenic (Fitzpatrick *et al*., 1995) and/or mutagenic properties, malachite green is banned in most of the developing countries (Marking *et al*., 1994). Formalin, a solution of 37% formaldehyde, is effective in treating *Saprolegnia* (Mitchell and Collins, 1997), and is the only fungicide registered for use in aquaculture (Marking *et al*., 1994). However, there are concerns about its affect on both the environment and personnel who handle it (Fitzpatrick *et al*., 1995). On the other hand, hydrogen peroxide appears to be a promising chemical for the treatment of *Saprolegnia* (Fitzpatrick *et al*., 1995; Marking *et al*., 1994) with minimal impact on the environment (Mitchell and Collins, 1997). But it is important to consider the species, life stage and water temperature while treating *Saprolegnia* with hydrogen peroxide (Rach *et al*., 1997). Contrary, in the present investigation, we formulated a simple treatment protocol that resulted in significant improvement and/or recovery from disease condition. Our results suggests that fishes at the beginning of infection treated in two steps i.e. dip treatment with 4g salt per litre of water for 2 min followed by with 5ppm $KMnO_4$ for 10 min resulted in faster recovery (Table 1).

In conclusion, the results of this study indicate that a combination treatment of salt (NaCl) and potassium permanganate ($KMnO_4$) might be an effective method to treat the fishes infected with *Saprolegnia* in mid hill altitude conditions and might be applicable in other species with minor modifications. Further, prospects of our low-cost technique might also be handy to the poor fish farmers who

Table -1: Changes in body weight, mortality and recovery from disease condition recorded between 0-6 weeks of treatments

Treatments	Numbers of fish treated (n)	Initial weight (g)	Final weight (g)	Mortality (n ;%)	Disease recovery (n ;%)
Control	30	6.1±2.9 *	7.1±0.9 *	1 (3) *	N A
A	30	5.9±3.1 *	6.2±2.5 **	15(50) **	4 (26) *
B	30	6.2±3.0 *	7.0±1.1 *	8 (26) ***	11(50) **

NA-Not applicable, Means without a common asterisks along the column significantly differ (P<0.05), Values presented are mean±SD.

have restricted access to the aquatic fungicide and/or other commercially available branded chemicals to be used for the treatment purposes.

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