RESEARCH COMMUNICATIONS

October 1993 to March 1994) for 16 h under supplemented light (30–40 μmol m⁻² s⁻¹) provided by three cool white fluorescent tubes. At 3-weeks stage the plants were exposed by opening the top of the polythene bag in the sachet method, and by removing the polythene cover in the other methods. The percentage establishment was recorded at 4-weeks stage and the growth of the plants was monitored periodically.

Among the two establishment media tried in the sachet method, the best plant (cv. Arka Neelamani) establishment was observed with EM1 (94%). Considerable rotting and mortality was noted with EM2 (47%) even while the sachets were closed, mainly due to poor drainage. Also, microbial growth was evident on the bits of farmyard manure and on the dying plant tissue. Therefore, further use of this medium was ruled out. Subsequent studies showed satisfactory plant establishment in EM devoid of soilrite or farmyard manure.

The present attempt to develop the sachet method for the acclimatization of grape plantlets was made as the efforts with other methods employing prostrays and mini pots, commonly practised by different laboratories and commercial establishments, proved less productive. The sachet method gave 90–100% establishment of ‘Arka Neelamani’ rooted plantlets in repeated trials. ‘Thompson Seedless’ and ‘Black Champa’ showed 90% and 95% establishment, respectively. The closed sachets by themselves acted as zero-energy humidity or mist chambers. The plants acclimatized by the sachet method were always more vigorous than those hardened by prostray and minipot methods (Table 1). This was contributed partly by the better microclimate around the plants.

Considerable high humidity was maintained around the plants even after the sachets were fully opened. When the ambient humidity was 30–35%, the humidity inside the open sachets one week after opening was 58–62%, while in the other two methods this was equal to ambient humidity. Further, the sachet acted as a barrier to air currents and this reduced the dessication effect as well as the water loss from the plant and the medium. This advantage was specific to the sachet method alone. Further, the plants in minipots and prostrays required transplanting to secondary nursery, which could altogether be avoided in the sachet method. The head space in the sachets provided sufficient scope for supplementing additional mixture; this avoided the need for a secondary nursery and provided considerable flexibility for bulking the hardened plants for field planting.

Employing the sachet method, the hardening of grape plantlets could be completed in 4 weeks and the plants could soon be shifted to either partial shade or full sunlight. It is suggested that the plants be kept in partial shade for sometime (1–2 weeks) before exposing them to full sunlight.

The sachet method has been found to work well for acclimatizing other tissue-cultured plants such as citrus and musk melon in our studies. This method will have far-reaching utility in commercial propagation of many horticultural plants through tissue culture.


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Pathogenic myxosporean infection in the early fry of Indian major carp, Catla catla

C. V. Mohan and K. M. Shankar
Department of Aquaculture, College of Fisheries, Mangalore 575 002, India

Here we present a case of pathogenic gill and kidney myxobolosis in the fry (15-day-old) of Indian major carp, Catla catla, and describe the histopathology and elucidate the mechanisms of pathogenesis. We also provide evidence to suggest that trophozoites and spores of the Myxobolus sp. could occur in the target tissue in less than 15 days following ingestion of infective spore.

Indian major carp hatchlings following yolk sac absorption (3–4 days post-hatch) are normally reared for up to one month in well-prepared nursery ponds. Nursery ponds with soil base are usually fertilized prior to stocking to encourage production of phyto and zooplankton, which are the preferred food items of carp fry.

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The information presented here is from a case study of mass mortality of 12–15-day-old Catla fry in a fish farm in Karnataka. Catla fry (12-day-old) stocked in nursery ponds were found to turn dark, become lethargic, swim in circles, settle at the bottom and die in large numbers. The entire stock of more than 1 million fry were dead within 3–5 days of appearance of the first clinical sign. Interesting findings from microscopical and histological investigations carried on live and moribund samples collected during the disease outbreak form the basis of this research communication.

Fresh examination of gill smears made from several moribund fry consistently showed the presence of large numbers of *Myxobolus* sp. spores (Figure 1) having the typical myxosporean spore features. Histological tissue sections of gills and kidney positively confirmed massive infection by the zoospore of *Myxobolus* sp. Large numbers of both spherical and irregular trophozoites and cysts of *Myxobolus* sp. were found in the brickell tissue of gill arches (Figure 2) and in the respiratory epithelial tissue of primary lamellae (Figure 3). The kidney was virtually replaced by the developing trophozoites of *Myxobolus* sp. (Figure 4). Longitudinal section of almost the entire length of the kidney showed very few kidney tubules and there was hardly any interstitial haematopoietic tissue in the anterior and middle kidney (Figure 5). The cysts in both the gills and the kidney were in different stages of development (Figure 6). Some had fully developed spores in the centre of the cyst, while the others were still in the developing vegetative phase.

Large-scale necrosis of the gill and kidney tissue by the developing trophozoites of *Myxobolus* sp. was the underlying reason for mass mortality of Catla fry. In the gills the developing cysts had destroyed the respiratory epithelial tissue and also had blocked branchial microcirculation. In the kidney the developing cysts had completely damaged and replaced the interstitial haematopoietic tissue and also had significantly reduced the number of functional renal tubules. Necrotic cellular and tissue damage in the kidney and gills would have significantly lowered the osmoregulatory, excretory, haematopoietic and respiratory efficiency and thus contributed to the observed massive mortality.

Catla fry start feeding immediately after yolk sac absorption (3 days post-hatch). The fry probably would have ingested the infective spores on day 3 or 4 post-hatch. Trophozoites and fully developed spores could be seen in 12–15-day-old Catla fry that were examined microscopically and histologically. The fact that fully developed spores could be seen in the gills and kidney implies that the *Myxobolus* sp. in question had completed its life cycle in less than 15 days following the ingestion of the infective spore by the fish.

The rapid development through vegetative phases into

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**Figure 1.** Fresh unstained preparation from gills showing large numbers of *Myxobolus* sp. spores (S). Note the presence of two polar capsules (arrowhead) and 1 sporoplasm (arrow). × 1000.

**Figure 2.** Photomicrograph showing the presence of large numbers of trophozoites of *Myxobolus* sp. (arrowed) in the gill connective tissue along the gill arch. H & E, × 100.

**Figure 3.** Photomicrograph showing developing trophozoites in the interlamellar region (arrowed) along the primary lamellae. H & E, × 100.
trophozoites and spore-containing cysts in less than 15 days accompanied by massive gill and kidney tissue damage could largely explain the high pathogenicity of *M. cerebralis*. The presence of cysts in various stages of development may also indicate that the fry were being continuously infected by the ingestion of infective spores present in the pond soil or in suspension.

The life cycle of myxosporea is largely controversial. Most authors suggest that the spores require a time for 'maturation' outside the host, probably in mud, before they become infective. Recently, it has been shown that *M. cerebralis*, *M. cotti* and *M. pavlovskii* require an intermediate host (Tubifex worm) inside which the spore develops and undergoes metamorphosis to become infective triactinomyxon or hexactinomyxon spore. Experimental infection studies with *M. cerebralis* in trout have shown trophozoites and spores to occur in the cartilage 40 and 90 days (respectively) after initial exposure to triactinomyxon spore at 16–17°C. Identical life cycle has been demonstrated for *M. cotti* and *M. pavlovskii*. In both species, following the ingestion of the infective spore, it has taken more than 120 days to produce a spore in the target tissue at 16–17°C. There is no information regarding the requirement of an intermediate host for myxosporeans of Indian freshwater fishes. Literature on the time taken by typical myxosporeans of Indian freshwater fishes to complete their life cycle is not yet clear. Experimental infection of Indian major carp hatchings and 10-day-old fry with *M. cerebralis* using spore-contaminated soil has been partially successful. Under experimental infection conditions, it was found to take 2 months or more following exposure to contaminated mud to produce the typical spore-containing cysts in the integument and gills at 28–30°C.

In view of the current knowledge on the life cycle of myxosporeans, the findings of the present study on the time taken to produce a spore in the target tissue following the ingestion of infective spore is very interesting and deserves further attention.

It is clear from the present observations that Indian major carp hatchings from the first feeding stage (3–4 days post-hatch) onwards can ingest spores from contaminated soil or those present in suspension and get the fatal infection. In addition, the present study has evidence to suggest that the infective spore can develop very rapidly in the target tissue following ingestion. In majority of sporozoans, the spores get released following the death of the infected fish or through rupture of cysts located at the surface. The spores can remain viable for several years in the pond soil. As there is no known effective systemic therapy for histozoic sporozoans in fish, it is vital to prevent the infection in nurseries by resorting to better management like...
drying and liming of nursery and rearing pond soil to kill the myxosporean spores.


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Anaerobes in the digestive tract of wood-eating grub Acanthophorus sp.

K. Sara Parwin Banu and K. Ramasamy
Department of Environmental Sciences, Tamil Nadu Agricultural University, Coimbatore 641 003, India

The presence of cellulyotic anaerobes and methanogens (H₂ oxidizers and acetate utilizers) in the gut of Acanthophorus larvae, which feed on citrus root, was observed. The cellulyolysers concentrate in the midgut and the methanogens were harboured in the hindgut. The cellulyolysers were mostly Clostridium sp. while the methanogens were fluorescent rod-shaped bacteria.

The digestive tract of the animal species normally paves a suitable niche for a variety of microorganisms to inhabit and multiply. Special attention is given to these gut microflora due to the fact that they play a major role in the host’s digestion, reproduction, excretion, etc.¹. The conditions prevailing in the gut favour the establishment of anaerobes which are also reported in insects. Substantial literature exists on the gut microflora of termites² and other xylaphagous insects like cockroaches³ and Orctes larvae⁴. But little is known on anaerobes in the gut of wood-feeding grubs like root-feeding Acanthophorus sp. or stem-feeding Batocera sp.

In the present research, the gut microflora of citrus root grub (Acanthophorus sp.) were examined. These grubs bore holes and feed on cellulose-rich bark and trunk. They plug the boreholes with the chewed frass material behind and hence reside under a partial anoxogenic condition. Due to this reason, the presence of anaerobes was suspected and assessed.

The grubs were collected from infected trees and reared under laboratory conditions on moistened sawdust separately up to last instar, which weighed about 50 g and was 10.5 cm in length. The last instar grub was anaesthetized in chloroform and dissected in an anaerobic hood under CO₂ stream to prevent the loss of anaerobes during dissection. The foregut, midgut and hindgut regions were incised separately and transferred to vials with modified Hungate’s broth⁵. The vials were sealed and flushed periodically with N₂ gas. The total anaerobes, cellulyolysers and methanogens were enumerated by Hungate’s roll tube technique using specific media⁶. The distribution of various anaerobes in different regions of the gut is given in Table 1. Degradation of the cellulosic substrates was observed through clearance in the opaque surrounding medium in the roll tubes. Filter paper degradation was also noted in the enrichments. Metabolic products of anaerobic cellulyolysis bacteria were assessed by gas chromatography⁷. Cellulyolysers were rod-shaped and mostly sporulating. Few of the purified isolates were characterized as Clostridium sp. The cellulyolysers converted the feed into acetate and propionate (Figure 1). Such volatile fatty acids production might support the host’s nutrition. Apart from strict anaerobes in the gut, microaerophilic cellulose-degrading bacteria were also observed in the posterior region of the hindgut.

Fluorescing methanogenic colonies isolated from the gut showed wide variation in their colony characteristics.

Table 1. Population (×10⁵ ml⁻¹) of anaerobes in the gut

<table>
<thead>
<tr>
<th>Anaerobes</th>
<th>Foregut</th>
<th>Midgut</th>
<th>Hindgut</th>
<th>Predominant cell shape</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total anaerobes</td>
<td></td>
<td></td>
<td></td>
<td>Rods, coccis</td>
</tr>
<tr>
<td>Cellulyolysers</td>
<td>ND*</td>
<td>4.2</td>
<td>1.1</td>
<td>Rods</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Methanogens</th>
<th></th>
<th></th>
<th></th>
<th>Predominant cell shape</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂ oxidizers</td>
<td>1.0</td>
<td>3.1</td>
<td>6.1</td>
<td>Rods</td>
</tr>
<tr>
<td>Acetate utilizers</td>
<td>2.5</td>
<td>2.0</td>
<td>3.0</td>
<td>Rods-forming filaments</td>
</tr>
</tbody>
</table>

*Not detected even in direct sample enumeration.

Figure 1. Growth and product profile of a cellulyolitic clostridium isolated from the gut of Acanthophorus grub.