ODA Project No. R5680

Final Report on Consultancy in Tropical Mycology

by

L G Willoughby

31 March 1994

Research Manager's Note

This consultancy, to Dr Willoughby of FBA Windermere (Retired), does not conform to the standard ODA contractual arrangements with Institutions, since Dr Willoughby is a superannuated retired scientist, who is Britain's only authority on the Saprolegniaceae. His work or method of working cannot be made to conform to the standard process of ODA contracts and reporting as he is unable to address himself to them or to understand their importance. He is a scientist of a bygone era.

His contribution is however essential to the resolution of the problems of EUS, the most important fish disease in the tropics and to the training of a successor. Thus he is used by the programme as well as he can be, under the constraints which his position, personality and age allow. This report reflects those constraints, and the primary concern that he maintain the culture collections of pathogenic strains, so essential to the work of the other scientists in this study.

R J Roberts

One year Consultancy in Tropical Mycology L G Willoughby

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Isolates of the *Aphanomyces* fungal pathogen, involved in EUS disease of fish, had been made in 1991 and 1992. However, these isolates, only three in number, had been made rather fortuitously. No firm recommendations could be given, as to how to isolate. However, in January 1993, the opportunity was taken to isolate from an excellent fish collection brought in to AAHRI Bangkok from Bangladesh by courtesy of ODA-AMOD, Dhaka. Several species of diseased fish were present in this collection and the pathogenic *Aphanomyces* was isolated from a half-beak (isolate BH), a rohu (isolate BR) and a snakehead (isolate BS). Isolation here proceeded in a logical sequence of stages, which bore in mind that the fungus in the fish has a very low viability. This viability is enhanced by the use of a liquid nutrient resuscitation system, kept largely bacteria-free by the use of the antibiotics penicillin-oxolinic acid. Small fungal colonies result and these are transferred to nutrient agar for final growth and isolation. The use of a second, different, antibiotic system here, penicillin-streptomycin, ensures that bacteria which became resistant in the liquid resuscitation, do not now survive. This isolation method has been written up and accepted for publication (Willoughby & Roberts, 1994).

Further work is projected on the EUS *Aphanomyces*, in the UK, and it is therefore necessary to continue to maintain the isolates here, in a culture collection. As it stands at present, EUS *Aphanomyces* is represented in this collection by isolates TA1, RF6, RF 6 Koch, RF 8 (from Thailand, LGW), M4, M6, M7 (from Thailand, J. Lilley), BH, BR, BS (from Bangladesh, LGW), 10D (from the Philippines, J Paclibare), 84-1240 (from USA, E J Noga). This is a total of 12. In addition, saprophytic, background *Aphanomyces* is represented by isolates

TF5, TF 41, TF 54, ASEAN 1, ASEAN 3 (all from Thailand, LGW), a total of 5. ASEAN 1 and ASEAN 3 can be named to species, as *A. laevis*. All of these isolates, both pathogens and saprophytes, are maintained in glucose-peptone-yeast extract broth, it having been shown that the cultures, if held on traditional agar slopes, have a very unpredictable long-term survivorship, for unknown reasons.

Fungi maintained in pure culture do not always retain their vital characteristics, and strains of *Fusarium* are notorious for their aberrant behaviour. However, the Saprolegniaceae, from past experience, appear to be more stable. But in this connection it is always advisable to use a minimal growth medium for sub-culture. Therefore it is unfortunate that the standard minimal glucose (only 0.3% w/v)-peptone growth medium for the Saprolegniaceae cannot be used for the EUS *Aphanomyces*, as discussed above; yeast extract must be added also. So far, comparative pathogenicity of the isolates, and whether or not their pathogenicity diminishes with the passage of time and repeated sub-culture, in the current regime, has not been studied. However, it was thought to be worth while to investigate their continued capacity to produce and release motile zoospores, the first step in the pathogenic process. Isolates of EUS *Aphanomyces*, made in January 1992 and in January 1993 were compared for their zoosporic capacity. In addition, isolate RF 6 original, 1992 and isolate RF 6 Koch postulates, 1993 were compared. The Koch postulates isolate had been used in successful fish challenge and then re-isolated from the fish. All of the isolates tested showed good zoosporic capacity and on this basis there seems no reason to doubt that vital characteristics are being retained. In the repeated sub-cultures, although growth rates are not being measured, it is obvious even to the naked eye that the pathogenic isolates continue to be slow-growing, in comparison with the saprophytic isolates of *Aphanomyces* from Thailand which are always fast growers.

Ulcers in diseased fish with EUS have *Aphanomyces* mycelium which is extremely wide in diameter. However, when the pathogenic *Aphanomyces* is isolated from these ulcers, onto traditional growth media, the cultured mycelium is much finer in diameter. This might cast doubt on the validity of the isolations:- has the right fungus been isolated? The explanation appears to be that the carbon/nitrogen (C/N) ratio of the growth medium can effect the mycelium morphology. With a high C/N ratio the fungus produces mycelium of narrow diameter, with a 'searching' habit. Presumably here, the excess carbon supplied is diverted into cell wall material (largely cellulose) and there is a high surface area/volume (s/v) ratio of the mycelium. With a low C/N ratio of the growth medium, the fungus produces mycelium of wide diameter, tending to an 'in-fill' habit. Presumably here, there is excess cytoplasm production and a low S/V ratio of the mycelium results. Low C/N ratios were set up by using ammonium chloride as the nitrogen source and also by using high protein, dead, fresh fish tissue or dead snake skin tissue as the nitrogen sources. 'In-fill' was particularly obvious in the two animal tissues and was so complete in the fish tissue that all traces of this disappeared completely. It is of great interest that the wide mycelium, 'in-fill' situation is also seen in the EUS ulcers, as mentioned above. Therefore it is concluded that the wide mycelium of *Aphanomyces invaderis* in the EUS ulcers, unexpected for an *Aphanomyces*, is merely a consequence of the background C/N status of the invaded fish tissue and not a hypertrophy condition arising from the parasitic interaction of fungus and host. These observations on growth in media of different C/N ratios help to explain the histopathological findings in diseased fish and have been written up for publication in the AAHRI Newsletter (Willoughby, 1994).

Presumably EUS infection of fish is initiated by spores (zoospores) of the pathogenic *Aphanomyces*. But nothing is known about the occurrence of these spores in natural waters

in Thailand and whether or not they are of seasonal occurrence. A complication in searching for them is that spores of saprophytic *Aphanomyces* will also be present, and, very likely, more numerous than those of the pathogen. However, at AAHRI in January 1994, a start was made in water analysis for *Aphanomyces* spores. Some difficulty was experienced in methodology and this was refined using local fish ponds at AAHRI and at ASEAN. Twenty one isolates of *Aphanomyces*, believed to have been driven from spores, were finally obtained from the two waters and all of them grew at 37°C, indicating that their status was saprophytic and not pathogenic, since this temperature is diagnostic. It was of great interest that when grown at 26°C these isolates showed a much wider range of growth rates than that seen hitherto for saprophytes. This work shows that *Aphanomyces* strains, derived from spores in water, are quite heterogeneous in their physiology; hopefully this heterogeneity can be exploited further, in the definition of a selective system to obtain the *Aphanomyces* pathogen only.

References

Willoughby L G (1994) *Aphanomyces invaderis*, the fungal pathogen of EUS, C/N ratios and morphogenesis. AAHRI Newsletter, in the Press.

Willoughby L G & Roberts R J (1994) Improved methodology for isolation of the *Aphanomyces* fungal pathogen of epizootic syndrome (EUS), in Asian fishes. Journal of Fish Diseases, In the Press.

UNIVERSITY OF STIRLING

FINANCIAL SUMMARY

R5680
Consultancy in Tropical Mycology

GRANT AWARDED	BUDGET 1993/94	ACTUAL 1993/94
Personal Emoulments	£10,400.00	£10,400.00
Bench fees	£1,000.00	£1,000.00
Consumables	£1,000.00	£960.86
Travel	£250.00	£258.00
Total	£12,650.00	£12,618.86

I certify that the expenditure detailed above has been actually and necessarily incurred in accordance with the terms of the Contractual Agreement dated 1st Feb 1990 .