Antibiotic effects of some aquatic hyphomycetes

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In vitro, antibiotic effects of the culture filtrates of 29 species of aquatic hyphomycetes against Gram-negative and Gram-positive bacteria, yeasts and hyphomycetes were studied. The culture filtrates of 38% of isolates were active in at least one of our tests for biological activity. Fifteen species inhibited growth of bacteria and four demonstrated antifungal activity. Filtrates of 35% of the isolates were antibacterial. About 50% of the isolates, those with the largest *in vitro* inhibitory activities (*Dimorphosphora foliicola*, *Flagellospora* sp. and *Mycocentrospora* sp.) were obtained from wood. Filtrates of *Articulospora tetracladia* were not only active against Gram −ve bacteria, but were also antiviral against bacteriophage T4 and influenza virus.

Antagonism between competing species has been reported in almost every ecological group of fungi (Gloer, 1995*a*). Aquatic hyphomycetes have not, however, been extensively surveyed as antibiotic producers, perhaps owing to their ecological peculiarities. There are a few papers describing intra- and interspecific interactions among aquatic hyphomycetes and their interrelations with aquatic ascomycetes involving sequestering of accessible resources, direct hyphal interference and production of diffusible inhibitory substances (Khan, 1987; Shearer & Zare-Maivan, 1988; Bärlocher, 1991). Chamier, Dixon & Archer (1984) reported inhibition of bacteria by aquatic hyphomycetes in field experiments. The first study on isolation and structural determination of antimicrobial compounds from *Anguillospora longissima* and *A. crassa* Ingold has resulted in the discovery of novel metabolites (Harrigan *et al*., 1995*a*, *b*). Quinaphthin, a new antimicrobial compound has been described from the aero-aquatic hyphomycete *Helicoon richonis* (Boud.) Linder (Fisher, Anson & Webster, 1988; Adriaenssens *et al*., 1994). Aquatic ascomycetes also remain relatively unexplored as sources of new antibiotic substances, although antagonistic activity involving production of such compounds has been detected in some species (Fisher & Anson, 1983; Asthana & Shearer, 1990; Poch, Gloer & Shearer, 1992).

Shearer & Zare-Maivan (1988) assumed that in lotic ecosystems any diffusible antibiotic substances secreted by fungi would be immediately washed away, so one might speculate that the aquatic environment cannot be selective for antibiotic producing organisms. Nevertheless, they reported that eight species of aquatic fungi (three of them aquatic hyphomycetes) were able to inhibit growth of other species at a distance, suggesting that they produce antibiotic substances.

We assume that the production of antibiotics by aquatic hyphomycetes, if any, would be relatively high because a significant proportion of secreted substances would be eliminated. On the other hand, for an antibiotic to be effective it would have to reach a certain threshold concentration (Fisher & Anson, 1983), at least in the close vicinity of hyphae.

MATERIALS AND METHODS

We assayed the antimicrobial activity of 92 monoconidial isolates of aquatic hyphomycetes (29 species) obtained from foam, submerged decaying leaves, wood, unidentified grasses or other substratum from Belarus watercourses. The following assay organisms were used: Gram −ve bacteria were *Escherichia coli* C 600, *Erwinia carotovora* subsp. *carotovora* 291-1, *E. carotovora* subsp. *atroseptica* 3-2, *E. herbicola* 37/15, *Pseudomonas aeruginosa* PA01, *P. fluorescens* VKM B-1474, *P. putida* M, *Xanthomonas campestris* VKM B-611/14, Gram +ve bacteria were *Bacillus cereus*, *B. mycoides*, *B. polymyxa* VKPM B-1130, *B. subtilis* VKPM B-1704, *Micrococcus luteus* and *Staphylococcus saprophyticus*. The bacteriophage was T4. Yeasts were *Candida utilis* (Henneberg) Lodder & Kreger, *Pichia anomala* (E. C. Hansen) Kurtzman, *Rhodotorula mucilaginosa* (A. Jörg.) F. C. Harrison VKPM Y-1020, *Waltomyces lipofer* (Slooff) Y. Yamada & Nakase VKPM Y-1001. Phytotoxicity tests were developed using *Chlorella vulgaris* Beyer 157. The foregoing strains were kindly provided by the Laboratory of Bacterial Genetics and the Teaching Collection, Department of Microbiology, Belarus State University. Hyphomycetes: *Alternaria alternata* (Fr.) Keissl., *A. panax* Whetzel, *A. solani* Sorauer, *Botrytis cinerea* Pers., *Cladosporium fulvum* Cooke, *Fusarium avenaceum* (Corda) Sacc., *F. oxysporum* Schltdl. var. *orthoceras* (Appel & Wollenw.) Bilaĭ, *F. sambucinum* Fuckel, *Ulocladium consortiale* (Thüm.) E. G. Simmons were from the Collection of the Department of Botany. Some of the above species are classical assay organisms and the rest were

Table 1. Inhibition of bacteria by the culture filtrates of aquatic hyphomycetes

	Source	N	Eco	Eca	Eat	Ehe	Pfl	Ppu	Xan	Bce	Bmy	Bpo	Bsu	Mlu	Ssa
Alatospora acuminata Ingold	l, w	3	—	±	—	?	—	—	—	—	—	—	—	?	—
Anguillospora longissima (Sacc. & Syd.) Ingold	l, u	10	—	—	—	?	—	—	±	—	±	—	—	±	±
Articulospora tetracladia Ingold	l	2	?	+	+	±	±	±	±	—	?	—	—	—	—
Clavariopsis aquatica de Wild.	l, u	7	?	±	±	—	—	?	±	±	+	+	±	±	±
Cylindrocarpon aquaticum (Sv. Nilsson) Marvanová & Descals	u	2	—	±	—	—	—	—	—	—	±	?	?	—	—
Dimorphospora foliicola Tubaki	w	2	?	±	±	?	+	+	±	—	+	?	?	?	—
Filosporella versimorpha Marvanová *et al.*	u	2	—	±	±	+	±	—	±	—	?	—	—	—	—
Flagellospora curvula Ingold	l	2	+	+	+	+	+	+	+	—	+	±	±	+	±
Flagellospora sp.	w	2	—	—	—	—	—	—	±	±	±	±	±	±	±
Lunulospora curvula Ingold	l	2	—	—	—	—	—	—	+	+	+	+	+	—	—
Margaritispora aquatica Ingold	l, u	4	—	±	?	—	±	—	—	—	±	?	?	±	±
Mycocentrospora sp.	l, w	4	—	—	—	—	—	?	+	+	+	+	+	±	±
Porocladium aquaticum Descals	f	2	—	?	—	—	—	—	±	±	±	?	±	?	—
Tetracladium marchalianum de Wild.	f, l	7	—	?	—	—	?	—	—	—	±	—	—	—	—
Vargamyces aquaticus (Dudka) Tóth	l	6	—	—	—	—	—	—	—	±	?	±	±	—	±

N = number of isolates tested.

Eco = *Escherichia coli*; Eca = *Erwinia carotovora* subsp. *carotovora*; Eat = *E. carotovora* subsp. *atroseptica*; Ehe = *E. herbicola*; Pfl = *P. fluorescens*; Ppu = *P. putida*; Xan = *Xanthomonas campestris*; Bce = *Bacillus cereus*; Bmy = *B. mycoides*; Bpo = *B. polymyxa*; Bsu = *B. subtilis*, Mlu = *Micrococcus luteus*; Ssa = *Staphylococcus saprophyticus*.

f = foam; l = submerged decaying leaves; u = unidentified grasses or other substratum; w = wood.

+ = Inhibition by all isolates of a particular species.

± = Inhibition only by some isolates of a particular species.

— = No reaction.

? = Questionable reaction (assay organism partly inhibited, weak or doubtful reaction, contradictory data from two experiments).

selected because of their great agricultural importance as phytopathogens.

The following liquid media were used in preliminary experiments for culturing the aquatic isolates: Czapek's mineral solution supplemented with 1% (w/v) of glucose as a carbon source, 2% (w/v) malt extract broth and potato broth prepared as for potato-dextrose-agar (PDA) (Hawksworth *et al.*, 1995) and then filtered and supplemented with 1% (w/v) of glucose or maltose; pH of all media was adjusted to 6·0. Results showed that the maximum antibiotic activity was achieved using the latter two media. Due to the slower utilization of maltose, and the possible repression of antibiotic synthesis by readily utilized sources of carbon (glucose), potato maltose broth was chosen for subsequent work.

Aquatic hyphomycetes were cultured in 250 ml flasks each containing 25 ml of medium without shaking at 20 ± 2 °C for 28 d. Flasks were inoculated with spore suspensions to obtain a count of 10^3–10^4 conidia/flask (varied according to species). After incubation, media were filtered through four layers of cheesecloth or decanted.

Culture filtrates were bioassayed using the well method. Four ml of 0·7% (w/v) nutrient agar containing approximately 10^6 bacterial cells or 4 ml of 0·7% (w/v) PDA containing 10^5 yeast cells or 10^4 conidia of filamentous fungi were spread over a layer of agar consisting of 12 ml of 1·5% (w/v) PDA in 90 mm diam. Petri dishes; pH of the media was 7·0 for bacteria and 6·0 for fungi. In the case of a preliminary antiviral test, the assay system contained bacteriophage T4 (10^7 per dish) and *E. coli* (10^6) to obtain a 'negative' lawn. In the phytotoxicity tests, the covering layer of agar (KNO_3 5·0 g; KH_2PO_4 0·3 g; $MgSO_4 . 7 H_2O$ 0·3 g; glucose 5·0 g; agar 15·0 g; distilled water 1000 ml; pH 6·5) contained 10^5 cells of *Chlorella*. One hundred µl of culture filtrate were placed in wells (8 mm diam.) in a lawn of assay organism and incubated at 37° (*E. coli*, *E. coli* + T4), 28° (other bacteria and yeasts), 20° (filamentous fungi) or at 25° with 12 h/12 h alternating light and dark cycles (*Chlorella*). The diameter of the zone of clearing (growth in *E. coli* + T4) was measured after 24 h (bacteria and yeasts), 72 h (filamentous fungi and *Chlorella*), 5 d (*C. fulvum*).

Antiviral activity was determined in collaboration with the Laboratory of Preclinical Study of Specific Activity of Viral Inhibitors, Belarus Institute of Epidemiology and Microbiology. Aquatic hyphomycetes were grown in Ranzoni's liquid medium (Thornton, 1963) supplemented with 1% (w/v) of glucose, the other conditions were as stated above. Cultures were filtered through a 'Millipore' membrane filter (0·22 µm pore size) and activity of the filtrate determined by the addition to the culture of chicken embryo fibroblasts which had been previously trypsinized and infected by the influenza virus (Votjakov *et al.*, 1986).

RESULTS

The ability to inhibit growth of certain bacteria was observed for filtrates of 52% of the aquatic hyphomycete species tested (Table 1). Isolates of *A. tetracladia* and *F. versimorpha* suppressed development of 6 and 5 of the 8 strains of Gram − ve bacteria, respectively. *Flagellospora* sp., *Mycocentrospora* sp., *L. curvula* and *P. aquaticum* inhibited growth of 6, 6, 4 and 3 of the 6 strains of Gram + ve bacteria and *X. campestris*, respectively. Culture filtrates of *C. aquatica*, *D. foliicola* and *F. curvula* were

Table 2. Inhibition of bacterial growth by the culture filtrates of some aquatic hyphomycetes (Mean ± S.E.M. of inhibition zone width (mm)). Width = (diam. of the zone of inhibition − diam. of the well)/2. Pooled data from two experiments (cultivations of aquatic hyphomycetes) each with three replicates (diameters of zones of inhibition). For abbreviations and conventions see Table 1. ND = not determined

Species & isolate no.	Source	Eco	Eca	Eat	Ehe	Pfl	Ppu	Xan	Bce	Bmy	Bpo	Bsu	Mlu	Ssa
A. acuminata 67	w	—	2·7 ± 0·2	—	?	—	—	—	—	—	—	—	?	—
A. longissima 13	l	—	—	—	—	—	—	2·8 ± 0·3	—	3·5 ± 0·6	—	—	1·3 ± 0·2	1·8 ± 0·2
A. tetracladia 20	l	?	6·8 ± 0·7	4·7 ± 0·3	6·2 ± 0·4	1·8 ± 0·3	?	1·3 ± 0·2	—	?	—	—	—	—
C. aquatica														
51	l	?	—	—	—	—	—	6·2 ± 0·6	6·2 ± 0·5	9·8 ± 0·7	6·5 ± 0·6	9·2 ± 0·6	1·8 ± 0·4	1·3 ± 0·2
52	l	?	?	?	—	—	—	3·2 ± 0·4	ND	6·5 ± 1·4	6·8 ± 0·6	8·2 ± 0·4	1·7 ± 0·3	?
79	u	—	2·7 ± 0·3	3·3 ± 0·4	ND	—	ND	?	—	2·7 ± 0·6	1·7 ± 0·2	?	—	—
C. aquaticum 55	u	—	3·0 ± 0·8	—	—	—	—	—	—	1·3 ± 0·3	?	?	—	—
D. foliicola 83	w	?	3·8 ± 0·4	6·5 ± 1·7	ND	2·7 ± 0·3	1·3 ± 0·3	3·2 ± 0·6	—	5·3 ± 0·7	?	?	?	—
F. versimorpha														
64	u	—	—	—	3·2 ± 0·3	—	—	—	—	?	—	—	—	—
65	u	—	4·2 ± 0·2	1·8 ± 0·4	2·7 ± 0·6	3·0 ± 0·5	—	3·3 ± 0·2	—	—	—	—	—	—
F. curvula														
60	l	2·0 ± 0·5	6·2 ± 0·4	2·5 ± 0·3	1·8 ± 0·2	2·2 ± 0·2	3·3 ± 0·4	2·2 ± 0·3	—	2·8 ± 0·2	4·7 ± 0·4	?	2·7 ± 0·4	2·7 ± 0·2
62	l	1·7 ± 0·3	7·3 ± 0·4	3·8 ± 0·2	2·2 ± 0·3	3·2 ± 0·2	3·8 ± 0·2	3·2 ± 0·3	—	5·2 ± 0·2	?	1·7 ± 0·2	5·7 ± 0·6	?
Flagellospora sp. 58	w	—	—	—	ND	—	ND	2·8 ± 0·2	5·2 ± 0·6	5·8 ± 0·2	5·3 ± 0·2	4·8 ± 0·4	5·7 ± 0·2	4·8 ± 0·2
L. curvula 103	l	—	—	—	—	—	—	2·2 ± 0·3	1·3 ± 0·2	2·7 ± 0·2	1·7 ± 0·2	1·2 ± 0·2	—	—
M. aquatica														
44	l	—	2·3 ± 0·6	?	—	1·7 ± 0·2	—	—	—	1·5 ± 0·3	—	—	2·8 ± 0·7	1·0 ± 0·3
77	u	—	—	—	ND	—	ND	—	—	3·3 ± 0·4	?	?	—	—
Mycocentrospora sp.														
63	w	—	—	—	—	—	—	15·2 ± 0·4	10·8 ± 0·2	12·8 ± 0·6	10·8 ± 0·2	11·5 ± 0·3	10·3 ± 0·2	10·2 ± 0·4
80	l	—	—	—	—	—	—	14·7 ± 0·2	10·2 ± 0·2	9·8 ± 0·4	10·3 ± 0·2	10·2 ± 0·2	8·3 ± 0·4	8·2 ± 0·2
P. aquaticum 70	f	—	?	—	—	—	—	3·2 ± 0·9	2·2 ± 0·4	5·5 ± 0·3	?	2·2 ± 0·2	?	—
T. marchalianum 8	l	—	?	—	—	—	—	—	—	3·2 ± 0·3	—	—	—	—
V. aquaticus 41	l	—	—	—	—	—	—	—	1·2 ± 0·2	?	1·7 ± 0·3	1·4 ± 0·2	—	3·9 ± 0·7

Table 3. Inhibition of yeast growth and conidia germination by culture filtrates of some aquatic hyphomycetes (Mean ± S.E.M. of inhibition zone width (mm)). Pooled data from two experiments (cultivations of aquatic hyphomycetes) each with three replicates (diameters of zones of inhibition). For conventions see Tables 1 and 2

Species & isolate no.	Source	Cut	Rmu	Wli	Aal	Apa	Aso	Uco	Cfu
C. aquatica									
51	l	1·8 ± 0·2	1·2 ± 0·2	2·7 ± 0·7	ND	ND	ND	ND	ND
52	l	1·3 ± 0·2	1·2 ± 0·2	2·2 ± 0·2	3·0 ± 0·6	3·2 ± 0·4	3·5 ± 0·4	?	?
C. aquaticum 55	u	—	?	—	3·3 ± 0·6	1·7 ± 0·3	?	3·3 ± 0·4	3·2 ± 0·3
Flagellospora sp.									
57	w	—	—	?	6·3 ± 0·4	6·7 ± 0·4	7·2 ± 0·2	5·2 ± 0·4	4·3 ± 0·3
58	w	—	—	3·3 ± 0·4	7·6 ± 0·4	5·3 ± 0·6	7·5 ± 0·8	6·5 ± 0·3	6·2 ± 0·4
Mycocentrospora sp. 63	w	—	—	10·8 ± 0·6	—	—	—	—	—

Cut = *Candida utilis*; Rmu = *Rhodotorula mucilaginosa*; Wli = *Waltomyces lipofer*; Aal = *Alternaria alternata*; Apa = *A. panax*; Aso = *A. solani*; Uco = *Ulocladium consortiale*; Cfu = *Cladosporium fulvum*.

active against some of the Gram + ve and Gram − ve organisms. None of the species tested inhibited the growth of *P. aeruginosa*. The following aquatic hyphomycetes were not biologically active (in parenthesis − number of isolates tested): *Anguillospora crassa* (1), *Clavatospora longibrahiata* (Ingold) Sv. Nilsson ex Marvanová & Sv. Nilsson (2), *Filosporella annelidica* (Shearer & J. L. Crane) J. L. Crane & Shearer (1), *Filosporella* sp. (3), *Heliscus lugdunensis* Sacc. & Thérry (3), *Lemonniera aquatica* de Wild. (7), *L. filiformis* R. H. Petersen ex Dyko (2), *L. terrestris* Tubaki (2), *Tetracladium setigerum* (Grove) Ingold (2), *Tricladium angulatum* Ingold (7), *T. splendens* Ingold (1), *Triscelophorus monosporus* Ingold (2), *Varicosporium elodeae* W. Kegel (1), *V. tricladiiforme* Roldán & Marvanová (1).

The filtrates of thirty-two isolates of aquatic hyphomycetes (35 %) were antibacterial. There were pronounced intraspecific differences in activity between isolates even with respect to the range of assay organisms inhibited (Table 2). Thus, all seven isolates of *C. aquatica* inhibited growth of Gram + ve bacteria and *X. campestris* to a different degree, but only isolate 79 demonstrated activity against *E. carotovora* and *E. carotovora* subsp. *atroseptica*. The isolates that caused the largest zones of inhibition were tested for phytotoxicity. Only the culture filtrate of *F. curvula* produced a zone of clearing of 4·8 ± 0·4 mm width (mean ± S.E.M.) on the *Chlorella* lawn.

Four species of aquatic hyphomycetes inhibited growth of yeasts, germination of conidia and subsequent hyphal growth (Table 3). In the case of filamentous fungi, however, after several days, growth resumed but at a much reduced rate and with the mycelium appressed to the agar. All species tested were unable to inhibit growth of *Pichia* and *Fusarium*, though some delay of development was observed in several combinations. Also, only some isolates of *C. aquatica* partially inhibited growth of *B. cinerea*. In some interactions, *A. longissima*, *L. curvula*, *M. aquatica* and *V. aquaticus* suppressed

development of *W. lipofer*, however, data from the two independent experiments were contradictory.

Culture filtrates of *A. tetracladia* 20, *D. foliicola* 82 and 83 induced zones of *E. coli* growth measuring $2{\cdot}2 \pm 0{\cdot}3$, $1{\cdot}7 \pm 0{\cdot}3$ and $3{\cdot}2 \pm 0{\cdot}6$ mm, respectively, around wells on the 'negative' lawn caused by phage T4. Further studies of the culture filtrate of *A. tetracladia* 20 gave a $14{\cdot}0 \pm 1{\cdot}0$ mm diam. inhibition zone of the influenza virus 'patches' development coupled with absence of a toxicity zone.

DISCUSSION

According to current concepts in aquatic mycology, the temporal dominance pattern of aquatic hyphomycetes suggests antagonistic interactions with bacteria and terrestrial fungi (Kaushik & Hynes, 1968; Chamier *et al*., 1984). Our experiments clearly demonstrated inhibition of bacterial and fungal growth by some aquatic hyphomycetes. Moreover, some genera of assay Bacterial (*Bacillus*, *Pseudomonas*) and all but one genus (*Ulocladium*) of fungi tested are typical constituents of aquatic biota or have been reported from water (Kaushik & Hynes, 1968; Bärlocher & Kendrick, 1974; Suberkropp & Klug, 1976; Chamier *et al*., 1984). It is known that antagonism *in vitro* is not always correlated with that in the field (Shearer & Zare-Maivan, 1988; Asthana & Shearer, 1990), but such correlations sometimes exist. An unidentified scolecosporous aquatic hyphomycete and *Tumularia aquatica* (Ingold) Descals & Marvanová were able to exclude competitors from colonized wood in field experiments (Shearer, 1992, 1993). The teleomorph of *T. aquatica*, *Massarina aquatica* J. Webster, produced diffusible antifungal substance(s) in laboratory experiments (Fisher & Anson, 1983). The most intriguing fact is that teleomorphs of *A. longissima* and *C. aquatica* also belong to *Massarina*. Thus, the ability to produce antibiotics seems to be a general characteristic of members of the genus and their anamorphs that inhabit aquatic environment.

Shearer & Zare-Maivan (1988) demonstrated that lignicolous aquatic ascomycetes and hyphomycetes are more antagonistic than foliicolous species because long-lasting substrata, such as woody debris, favour colonization by species which are capable of defending captured resources, in particular, by the production of antimicrobial compounds (Wicklow, 1981). Along with aquatic ascomycetes, *C. aquatica*, frequently occurring on wood, inhibited growth of 23 out of 25 fungal species tested (Shearer & Zare-Maivan, 1988). It also showed high antibacterial and some antifungal activity in our experiments. Culture filtrates of 7 of the 10 isolates obtained from wood were antimicrobial in the present study. The isolate of *A. longissima* described by Harrigan *et al*. (1995*a*), and antagonistic isolates of ascomycetes (Asthana & Shearer, 1990; Poch *et al*., 1992) were also from wood.

Some correlation between the production of pigments and the level of antibiotic activity has been noted in aquatic ascomycetes (Asthana & Shearer, 1990). The production of diffusible pigments is also known for *M. aquatica* (Fisher & Anson, 1983) and *T. marchalianum* (Aimer & Segedin, 1985) which were found to be antagonistic, and was observed in our experiments in the culture filtrates of *C. aquatica* (yellowish staining of liquid media), *Flagellospora* sp. (olive green, poorly water soluble substance), *M. aquatica* (reddish), *Mycocentrospora* sp. (black), *P. aquaticum* (reddish brown).

The activity against Gram −ve bacteria and antiviral effects of the culture filtrates of *A. tetracladia* are of particular interest. To our knowledge, it is the first aquatic fungus to be reported as having antiviral activity.

Some recent screening programmes involving investigation of fungi inhabiting specific ecological niches have led to the discovery of new biologically active isolates and metabolites. In Petri dish assays of over 150 mainly slower growing coprophilous fungi (Gloer, 1995*a*), approximately 60% have displayed antifungal effects at a distance indicating the involvement of antibiotic substances. More than 50% of the active strains also produced antimicrobial metabolites when grown in liquid culture. The percentage of new bioactive compounds isolated through these studies were unusually high (58%). Screening of 12 species of nematophagous fungi for antimicrobial and nematicidal activity (Anke *et al*., 1995) has resulted in 7 species with antibacterial and antifungal effects. The diameters of inhibition zones (e.g. activity) in the disc diffusion assay were nearly comparable to those obtained in our experiments. Each disc corresponded to 2 ml of culture filtrate, however, whereas we assayed only 0·1 ml using the well method.

It is thought that aquatic hyphomycetes, due to their specific habitat, may have biosynthetic capabilities different from those of terrestrial fungi. Studies of their secondary metabolites could, therefore, result in the discovery of new natural bioactive products of medical and agricultural importance (Gloer, 1995*b*). Data from our experiments suggest that certain isolates of aquatic hyphomycetes secrete antibiotic substances. It should also be noted that some antibiotics could be associated with mycelium and, in this case, they would have remained undetected in the present study.

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