Fungal Diversity

Nematicidal effect of freshwater fungal cultures against the pine-wood nematode, *Bursaphelenchus xylophilus*

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Twenty-two filtrates and 13 water-soluble extracts of broken fungal mycelia from 130 freshwater fungal cultures were found to be pathogenic to the pine-wood nematode, *Bursaphelenchus xylophilus* following 48 hours exposure *in vitro* screening. The mobility of over 90% of nematodes were inhibited by filtrates from *Ophioceras commune* (97.18%), *Pseudohalonectria adversaria* (96.49%), *Pseudohalonectria lignicola* (96.15%), *Massarina thalassioidea* (93.2%), *Caryospora callicarpa* (95.2%) and *Annulatascus* sp. (96.12%) and the mycelia extracts from *Helicomyces roseus* (98.95%), *Phomatospora berkeleyi* (94.96%) and *Pseudohalonectria lignicola* (95.59%). Aliphatic extracts of four freshwater fungal solid state fermentation products were found to immobilize over 50% of nematodes within a 12 hour exposure period at a concentration of 40 mg/mL. It was also observed that the aliphatic extracts of *Pseudohalonectria adversaria*, *Xylaria* sp. and *Hyphomycete* sp. were nematicidal, whereas *Massarina bipolaris*, *Caryospora callicarpa* and an unidentified strain were found to be narcotic in nature because nematodes revived when they were transferred to sterilized water. When screening for nematicidal activities it is important to use approximate neutral and saline environments similar to the natural habitats of the test nematodes, as nematodes can be affected by extreme pH and high osmotic pressure.

Key words: aliphatic extracts, broken mycelia extracts, filtrates, pathogenic

Introduction

Searching for new microbial strains as sources of biological nematicides is an important goal for those seeking to reduce the significant economic damage caused by plant-parasitic nematodes. Fungi exhibit a range of specificities and modes of action in their antagonistic activities toward nematodes, offering an extensive pool of potential candidates to test (Siddiqui and Mahmood, 1996). Like other microbes, fungi can directly parasitize

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nematodes or secrete nematicidal metabolites or enzymes that affect nematode viability. Toxic and inhibitory effects of several fungal filtrates have been confirmed (Desai *et al.*, 1972; Alam *et al.*, 1973; Sing *et al.*, 1983; Khan and Hussain, 1989; Khan and Kgan, 1992; Chattopadhyay and De, 1995; Pathak and Kumar, 1995; Sankaranarayanan *et al.*, 1997). One interesting selective nematicide reported recently is omphalotin, a cyclic dodecapeptide isolated from *Omphalotus olearius*. Omphalotin is very potent against the plant parasite *Meloidogyne incognita* (LD_{50}: 0.75ug/mL). It is considerably more active than the commercially available nematicide ivermectin (Mayer *et al.*, 1997; Stemer *et al.*, 1997).

The objectives of this study were to determine the pathogenic effect of freshwater fungi isolated during our study on fungi on submerged wood in streams (Cai *et al.*, 2002; 2003) on the pine wood nematode, *Bursaphelenchus xylophilus*.

Materials and methods

Fungi

One-hundred and thirty fungal strains (most belonging to the genera *Annulatascus*, *Camposporium*, *Caryospora*, *Cyathus*, *Diaporthe*, *Dictyosporium*, *Dyrithiopsis*, *Eutypa*, *Eutypella*, *Leptosphaeria*, *Massarina*, *Nectria*, *Ophiobolus*, *Ophioceras*, *Phomatospora*, *Pseudohalonectria*, *Savoryella*, *Torula* and *Xylaria*) were isolated from the submerged woody substrates collected in various freshwater habitats (e.g. Cai *et al.*, 2002, 2003). All strains were maintained on potato-dextrose agar slants.

For submerged cultivation, fungi were inoculated into 250 mL Erlenmeyer flasks each containing 70 mL medium (2% soybean power, 2% glucose, 0.2% peptone, 0.5% starch, 0.2% yeast cream, 0.4% NaCl, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% $CaCO_3$) and incubated for 10 days at 200 rpm at 26°C on a rotary shaker. The culture filtrates and the water-soluble extracts of broken hyphae respectively served as stock solutions for screening nematicidal activity. When the pH of the submerged cultures was over 8 or under 5 the mobility of the nematodes were adversely affected. The influence of pH on nematicidal properties was considered when adjusting the stock solutions to neutral with NaOH and HCl solutions. Freshly prepared sterilized media were clarified by centrifugation and designated as a control.

For solid-state cultivation, fungi were inoculated into 1000 mL Erlenmeyer's flasks containing wheat medium. Wheat medium was prepared by soaking wheat seed in tap water for 3 days. The water was decanted, and *ca.* 200 g of wheat was packed loosely in individual Erlenmeyer's flasks. A cotton

plug was added and the flask was autoclaved twice at 15 psi for 40 minutes. Mycelial plugs (1cm) were transferred to the flasks from the growing margin of the fungal colony on PDA plates and incubated at 27°C till the fungus colonized the entire mass of wheat seed. The contents of flasks were transferred to a shallow tray and lyophilised. The dried fungal hyphae was ground to powder using a mortar and pestle. The dried powder was soaked with $CHCl_3$ and MeOH solvents (1:1, v/v), maintained at room temperature for a week and filtered. The solvents were removed in a Vacuo at temperatures not above 70°C and the aliphatic extract of fungal mycelia was obtained and dried in a desiccator. A fixed amount of the well-dried extract was dissolved in DMSO, the concentration of which never exceeded 3% in the tested solution and was diluted with water containing 0.3% (v/v) Tween-20 to prepare stock solution of 40mg/mL. The same amount of DMSO dissolved in water containing 0.3% (v/v) Tween-20 was used as a control.

Nematodes

Botrytis cinerea was cultured on potato dextrose agar in Petri-dishes (diam. 90 mm) at 26°C. Petri-dishes with fully grown fungus were inoculated with *Bursaphelenchus xylophilus* and left until fungal mycelia were completely consumed. The cultured nematodes (mixed stage) were separated from the culture medium by the Baerman funnel technique and enumerated on a grid under a microscope (× 20). An aqueous suspension of the nematode (*ca.*15 000 nematodes per mL) was prepared by appropriate dilution for use as a working stock.

Bioassay of nematicidal activity

The nematotoxin bioassay was made in 6 cm Petri-dishes. Three hundred nematodes in 20 µL of aqueous suspension was transferred to Petri-dishes containing 2 mL of fungal extracts and gently mixed. All dishes were incubated at 25°C. The numbers of live and inactive nematodes were counted under a binocular microscope after different incubation times. Nematodes were considered dead if they gave no response to physical stimuli such as mechanical stirring and pricking with the point of a needle. Toxicity was estimated according to the mean percentage of dead nematodes. Each treatment was replicated four times and the data obtained analysed.

The revival rate of immobilized nematodes after treatment by fungal aliphatic extracts was also established. After 36 hours, the nematodes whose mobility was inhibited in the extracts were transferred to tubes and centrifuged at 10000 rpm. The supernatant fluid from each tube was removed with a

pipette leaving about 20 µL. Sterilized water was added to make a total volume of 1.5 mL. The tubes were again centrifuged at 10000 rpm. The supernatant fluid was removed and the nematode-containing solution remaining at the bottom was pipetted into a Petri-dish containing 1 mL sterilized water. The proportion of revived nematodes was estimated after 12 hours in water.

Where there were no apparent effects on the nematodes during the exposure periods, the fungi were usually omitted from further studies. Experiments were treated in triplicate for the fungi that appeared to immobilize over 50% of nematodes.

Results and discussion

Toxic effect of submerged fungal cultures on Bursaphelenchus xylophilus

Over 50% of *Bursaphelenchus xylophilus* individuals were immobilized in 22 fungal filtrates (Table 1). After 48 hours, over 90% of nematodes were immobilized in filtrates of *Ophioceras cummune* (97.18%), *Pseudohalonectria adversaria* (96.49%), *Pseudohalonectria lignicola* (96.15%), *Massarina thalassioidea* (93.2%), *Caryospora callicarpa* (95.2%) and *Annulatascus* sp. (96.12%).

Over 50% of *Bursaphelenchus xylophilus* were immobilized in a solution of 13 broken fungal mycelia (Table 2). After 48 hours, a maximum death of 98.95 percent of individuals were recorded using isolates of *Helicomyces roseus*, followed by *Pseudohalonectria lignicola* (95.59%) and *Phomatospora berkeleyi* (94.96%).

The nematicidal effect of on nematodes can be group into five categories (Table 3).

In the first category, the nematicidal effects occurred in both crude filtrates and water-soluble extracts of broken hyphae. When the pH of the test extracts were adjusted to pH 7.0, their was decreased in immobilization. For example, the crude filtrate of *Ophioceras commune* immobilized 52% of nematodes within 48 hours of exposure, but the same filtrate at pH 7.0 immobilized 0.63% of nematodes. It is thought that extremes in acidity or alkalinity result in the death of *B. xylophilus* because nematodes require appropriate pH values for survival. Therefore, when extracts are tested for nematicidal activity, the pH value of the extracts are important.

Table 1. Effect of fungal filtrates on the pathogenicity of *Bursaphelenchus xylophilus in vitro*.

Strain	Name of fungi	Exposure time (fungal filtrates) in hours		
		12	24	48
47	*Aniptodera* sp.	59.63	69.33	52.75
54	*Aunulatascus* sp.	65.79	93.68	96.12
104	*Caryospora callicarpa*	57.19	83.03	75.11
107	*Caryospora callicarpa*	25.69	93.10	95.2
92	*Diaporthe* sp.	3.33	10.80	63.79
91	*Diaporthe* sp.	53.18	81.16	78.98
38	*Helicomyces roseus*	30.70	79.85	82.46
43	*Helicomyces roseus*	48.51	83.67	78.89
42	*Helicomyces roseus*	70.29	75.16	77.91
95	*Leptosphaeria* sp.	70.11	78.08	80.26
96	*Leptosphaeria* sp.	-0.36	0.69	70.41
60	*Massarina bipolaris*	43.67	73.62	85.59
53	*Massarina thalassioidea*	48.60	91.70	93.20
69	*Ophioceras commune*	70.21	79.34	97.18
83	*Paraniesslia* sp.	65.10	79.80	85.33
130	*Pseudohalonectria adversaria*	81.60	85.86	87.27
129	*Pseudohalonectria adversaria*	81.22	85.54	85.67
126	*Pseudohalonectria adversaria*	69.59	86.20	89.44
77	*Nectria* sp.	78.65	85.32	96.49
100	*Pseudohalonectria lignicola*	88.28	95.44	96.15
16	Unidentified fungus	49.36	62.20	75.27
14	Unidentified fungus	56.69	74.36	71.92

In the second category, the fungi had no apparent effect on the vitality of *B. xylophilus* immersed in sterilized filtrates and hyphal extracts for periods up to 48 hours. However, when living nematodes were placed in neutral stocks adjusted with acid or alkali, many of them became inactive and appeared to have been killed by substances in the filtrates. Immobilization of the nematodes may have been caused by high ion concentrations because *B. xylophilus* may not survive in high ionic environments. For example, the neutral filtrate of *Torula herbarum* 123 immobilized up to 75% of nematodes within 48 hours, but the unadjusted filtrate immobilized only 23%. The alkaline-treated hyphal extract of *Ophioceras cummune* 66 immobilized up to 60% of nematodes, whereas the crude extract immobilized only 14% of them. Therefore when the nematicidal activity of solutions is tested, osmotic pressure should be considered and the activity should be evaluated in approximate ionic environments.

Table 2. Effect of fungal broken mycelia solution on the mortality of *Bursaphelenchus xylophilus in vitro*.

Strain No.	Name of fungi	Exposure time (fungal broken mycelia) 12h	24h	48h
31	*Annulatascus triseptata*	3.51	47.17	81.86
33	*Annulatascus triseptata*	20.57	95.06	77.30
103	*Caryospora callicarpa*	2.18	70.63	56.97
7	*Cyathus pallidus*	56.38	63.54	53.05
42	*Helicomyces roseus*	89.73	73.51	98.95
65	*Ophioceras commune*	18.60	93.58	71.91
81	*Paraniesslia* sp.	60.99	97.13	82.76
115	*Phoma* sp.	59.41	70.01	61.62
74	*Phomatospora berkeleyi*	17.01	95.38	94.96
93	Unidentified fungus	36.36	26.36	79.2
28	Unidentified fungus	59.32	66.01	68.24
100	*Pseudohalonectria lignicola*	84.10	88.02	95.59
14	Unidentified fungus	55.69	65.24	51.91

Table 3. The five postulated circumstances on the nematicidal reasons of fungi.

Postulated reasons for immobilizing nematodes	Crude filtrates	Neutral filtrates	Crude hyphae	Neutral hyphae	Number of fungi belong to this type
Improper acidic and alkaline environment	+	-	+	-	26
Over high osmotic pressure	-	+	-	+	32
Exotoxin	+	+	-	-	19
Endotoxin	-	-	+	+	13
Exotoxin and endotoxin	+	+	+	+	3

+: Over 50% of *B. xylophilus* were immobilized after 48 hours exposure time
-: Less 50% of *B. xylophilus* were immobilized after 48 hours exposure time

In the third category, the nematicidal effect only occurred in the filtrates and not in the broken fungal hyphal extracts. It was concluded that the nematicidal effect resulted from toxic substances secreted outside the fungal hyphae, i.e. exotoxins. Twenty-two fungal filtrates immobilized the nematodes, not only in crude filtrates but also in filtrates adjusted to pH 7.0. These fungi were therefore subjected to further study. The most potent fungi were species of *Caryospora*, *Diaporthe*, *Leptosphaeria*, *Massarina*, *Ophioceras* and *Pseudohalonectria* (Table 1).

Table 4. Effect of different fungal hyphae extracts on immobilization of *B. xylophilus*.

Strain	Genus	Exposure time (hours)		
		12	24	36
1a	Hyphomycetes sp.	86.9	85.5	94.5
5a	*Massarina bipolaris*	9.1	23.4	82.4
5c	*Massarina bipolaris*	7.6	78.0	96.9
14	Unidentified fungus	6.9	53.4	87.1
17	Unidentified fungus	6.7	10.8	87.2
28	Coelomycetes sp.	28.7	32.3	27.2
47	*Aniptodera* sp.	7.7	58.8	96.8
50	*Massarina thalassioidea*	65.6	79.7	49.2
56	*Ophiobolus* sp.	8.8	10.2	11.4
81	*Paraniesslia* sp.	15.6	19.6	30.3
83	*Paraniesslia* sp.	9.7	12.4	82.3
84	*Paraniesslia* sp.	85.2	86.4	69.1
91	*Diaporthe* sp.	11.5	14.2	33.8
93	Unidentified fungus	31.2	93.3	61.9
103	*Caryospora callicarpa*	12.1	16.5	86.1
108	*Camposporium quercilola*	9.4	17.8	18.4
109	*Camposporium quercilola*	8.1	8.2	7.5
110	*Camposporium quercilola*	7.8	23.7	89.3
115	*Phoma* sp.	6.4	52.1	95.5
120	*Pseudohalonectria adversaria*	97.0	62.0	50.0
128	*Xylaria* sp.	7.8	9.2	92.0
129	*Pseudohalonectria adversaria*	5.3	9.0	98.2
130	*Pseudohalonectria adversaria*	8.3	12.5	25.6

In the fourth category, the nematicidal effect only occurred in the broken hyphal extracts and not in filtrates. The nematicide is secreted within the fungi hyphae (i.e. endotoxin). Natural and neutrally adjusted extracts of 13 fungal hyphae were pathogenic to the nematodes. They were species of *Annulatascus*, *Caryospora*, *Cyathus*, *Ophioceras* and *Phomatospora*. Among them, 8 strains immobilized up to 70% of nematodes within 48 hours exposure and 4 strains up to 90% within 48 hours (Table 2).

In the fifth category, the nematicidal effect was most interesting because the fungal strains caused nematodes to die as a result of intracellular (endotoxins) and extracellular metabolites (exotoxins). Only three strains produced broken mycelia and culture filtrates that affected up to 50% of the nematodes within 48 hours of exposure. They were *Helicomyces roseus*, *Pseudohalonectria lignicola* and the unidentified fungus No.14 (Tables 1 and 2).

Toxic effect of freshwater fungal solid-state fermentation products on B. xylophilus

The effect of various extracts on the pine wood nematode is summarized in Table 4. The pathogenicity to nematodes did not always increase with length of exposure to the fungal extracts. After 12 hours the pathogenicity varied from 5.3 to 97%. The maximum pathogenicity of 97% occurred in the *Pseudohalonectria adversaria* isolate, followed by the Unidentified strain No. 1a (86.9%), *Paraniesslia* sp. (85.2%) and *Massarina thalassioidea* (65.6%). No nematode mortality was observed even after 36 hours exposure in extracts of *Camposporium quercilola*, *Diaporthe* sp., *Coelomycetes* sp., *Ophiobolus* sp., *Paraniesslia* sp. and *Pseudohalonectria adversaria*.

It was also observed that effects of culture extracts on nematodes mobility varied with length of exposure time. In the first example, as illustrated in the treatment of *Camposporium quercilola*, the nematodes were unaffected and moved at each observation and activity levels did not diminish with time. In second case the nematicidal activity of the culture extracts of *Pseudohalonectria adversaria* 129, *Paraniesslia* sp. 83, *Caryospora callicarpa*, the unidentified strains No. 17 and No. 1a increased with exposure time. In the third case, nematicidal activity decreased with time. For example, over 90% of nematodes were immobilized with initial exposure in *Pseudohalonectria adversaria* 120 and 93 mycelial extracts, but at least 30% of those nematodes recovered after further exposure. In the fourth case, e.g. extract of *Massarina thalassioidea* 50, the nematodes were initially immobilized and became inactive after 24 hours of exposure, but recovered during the remainder of the exposure period. In the fifth cases, the number of immobilized nematodes was relatively stable from the start to the end of exposure period.

The results of studies with *Pseudohalonectria adversaria* 120, 129 and 130 and *Paraniesslia* spp. 81, 83 and 84 were equivocal. The reasons for these inconsistent results are unknown, however a possible explanation is intraspecific differences in the fungal strains. The various strains may produce different amounts and types of active metabolites.

Revival of immobilized nematodes

The number of revived nematodes was investigated for a further 12 hours in water in stock solutions (See Table 5). Results from strain No. 103, No. 17 and No. 5a showed almost all of the nematodes recovered their mobility and became fully active after a further 12 hours in water, whereas extracts of

strains No. 129, No. 128 and No. 1a appeared to kill the nematodes. In treatments with strains No. 129, No. 128 and No. 1a, the nematodes did not recover from exposure to extracts and less than 10% were active after immersion in water for 12 hours. Study of reversibility of the toxic effects of funal extracts indicate that *Pseudohalonectria adversaria* 129, *Xylaria* sp. 128, and *Hyphomycetes* sp. 1a were nematicidal, whereas *Massarina bipolaris* 5a, *Caryospora callicarpa* 103 and an unidentified strain No. D17 were possibly narcotic in nature because nematodes revived when they were transferred to sterilized water.

Table 5. Results of the reversibility of the nematicidal toxin.

Strain No.	1a	129	128	115	110	103	47	17	14	5a	5c
Pathogenicity (% in aliphatic extracts)	94.5	98.2	92.0	95.5	89.3	86.1	96.8	87.2	87.1	82.4	96.9
Pathogenicity (% in sterilized water)	96.7	96.9	92.8	54.6	74.9	21.3	76.5	24.2	62.8	30.6	62.8
Revival rate (%)	-2.2	1.3	-0.8	40.9	14.4	64.8	20.3	63.0	24.3	51.8	34.1

As compared to previous studies (e.g. Alam *et al.*, 1973; Khan and Hussain, 1989; Chattopadhyay and De, 1995; Pathak and Kumar, 1995; Sankaranarayanan *et al.*, 1997), this study has shown that nematicidal effects of fungi extracts are unstable and the dead nematodes immersed in stock samples can be revived with increasing exposure time. For example, the broken mycelia solution of *Ophiobolus* sp. 56 immobilized the nematodes up to 49.76% within 24 hours exposure period, but only 1.58% over a further 24 hours exposure. The possible reason is that the toxicity of fungal metabolites is too low to kill the nematodes, that is, the nematodes are immobilized by the solutions, but recover with increasing time.

In conclusion, our results indicate that fungal extracts have potential to be developed as the nematicides, but the use of fungal extracts to control the pine-wood nematodes needs further study before being used as an alternative to chemical control. The main factors to be considered are fungal mass production and phytotoxicity.

Why do freshwater fungi produce nematicides

In freshwater ecosystems, submerged woody substrata are main energy input (Wong *et al.*, 1998). Wood is, however, a substrate greatly deficient in nitrogen and therefore the nitrogen utilised by freshwater fungi may be

obtained from other sources. Nematodes, are cosmopolitan organisms, adapted to living in soils and water. They have been shown to be an integral part of various ecosystems, serving as food for small invertebrates or fungi (Dropkin, 1980). With their high nitrogen component, nematodes are thought to play an important role in providing nitrogen to other organisms in freshwater ecosystems. Several nematophagous fungi have previously been reported from wood submerged in freshwater, e.g. *Dactylella ellipsospora* Grove (Hyde and Goh, 1998) and *Dactylella aquatica* (Ingold) Ranzoni (Kane *et al.*, 2002), and these species are normally found from the dead bodies of the nematodes (K.D. Hyde, pers. comm.). It would also make sense if other wood inhabiting fungi occurring on wood in freshwater were able to supplement their diets by obtaining nitrogen via digesting nematodes. The ability for these fungi to produce nematicides that can kill nematodes, which they can subsequently consume, would be advantageous. A pine-wood nematode, *Bursaphelenchus xylophilus* was therefore used in this study to establish whether freshwater fungi can produce nematicides and the results are positive. Further studies are necessary to establish whether freshwater fungi can kill and utilise nematodes in nature and whether any of these nematicides have biotechnological potential.

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