Scientific Reports of the Faculty of Agriculture
Okayama University Vol. 85, 1-6 (1996)

Screening of Fungal Cultures for Nematicidal Activity and Preliminary Fractionation of a Nematicidal Fungal Culture

Kazuyoshi Kawazu, Sun Jian Hua[a] and Hiroshi Kanzaki
(Department of Bioresources Chemistry)

Cultures of 99 strains of fungi in potato-sucrose-malt extract medium were screened for nematicidal activity. Strong activity was found in the supernatant of the culture of fungus 18. Activity-guided preliminary fractionation of the supernatant showed that the active compound was neutral and water soluble.

Key words : nematicidal activity, screening of fungal cultures, *Bursaphelenchus xylophilus*

Introduction

We have on the earth many species of nematodes which are parasites of animals or plants. Plant parasitic nematodes cause serious damage to agricultural crops. For the purpose of defending crops against nematodes' attack, it is desirable to devise potent and practical nematicidal agents.

The first of the authors devised a novel bioassay method[1], the so-called "cotton ball on fungal mat method", using the pine wood nematode *Bursaphelenchus xylophilus* as a test nematode, and searched with this assay method for novel nematicidal compounds in higher plants, imperfect fungi, and actinomycetes to find several active compounds, e.g. 9,10-epoxy-heptadec-16-ene-4,6-diyn-8-ol[2], nucleoside antibiotic dehydrosinefungin[3], and cyclodepsipeptides bursaphelocides A and B[4].

This paper deals with the screening of fungal cultures for nematicidal activity, and preliminary fractionation of an active culture.

Materials and Methods

Test nematodes

The pine wood nematodes, *Bursaphelenchus xylophilus* were collected by the Baermann funnel method from wood chips of the trunk or stem of wilted pine trees in Handayama experimental forest, Okayama University, and subcultured repeatedly for a decade by being fed on the fungus *Botrytis cinerea* grown on glucose Czapek-Dox agar medium. For the bioassay, a suspension of 15000 heads of the nematode in 1ml of sterilized water was prepared.

Fungal strains screened

Ninety-nine strains of fungi were isolated from soil samples, and the cultures were maintained as slants in potato-sucrose-malt extract(PSM)-agar medium (agar 1.5%) before screening.

Media examined for the selection of the best mass-production condition

PY medium (pH 7.0)[5]: starch 10.0, glycerol 10.0, glucose 5.0, meat extr. 5.0, Polypepton 3.0, yeast extr. 2.0, caseine 1.0, calcium carbonate 2.0, thiamine 0.01 (g/l water).

PSM(potato-sucrose-malt extract) medium (pH 5.5-6.5): peeled potato 300, sucrose 20, malt extr. 2 (g/l water).

YM(yeast extract-malt extract) medium (pH 7.3): yeast extr. 3.0, malt extr. 3.0, Polypepton 5.0, glucose 10.0 (g/l water).

Received October 4, 1995

a) *Visiting research worker for 1 year (1994. 10-1995. 10) from Tianjin Normal University, the People's Republic of China*

CM medium (pH 7.0)[5]: glucose 10.0, glycerol 5.0, corn steep liquor 3.0, meat extr. 3.0, malt extr. 3.0, yeast extr. 3.0, calcium carbonate 2.0, thiamine 0.01 (g/l water).

Y-3 medium (pH 7.2)[6]: glucose 10, yeast extr. 5, Bacto Tryptone 5, malt extr. 10 (g/l water).

Glucose Czapek-Dox medium (pH 5.8-6.0): sodium nitrate 2.0, dipotassium hydrogen phosphate 1.0, magnesium sulfate hydrate 0.5, potassium chloride 0.5, ferric sulfate hydrate 0.01, glucose 36 (g/l water).

Bennett's medium (pH 7.3): glucose 10, yeast extr. 1, beef extr. 1, Polypepton or N-Z-amin A 2 (g/l water).

No.2 medium (pH 7.3): glutinous starch syrup 40, soyaflour 20, Pharmamedia 10, Sungrain 5, soybean oil 3, calcium carbonate 3, ferric sulfate hydrate 0.01, cobalt(II) chloride hydrate 0.001, nickel(II) chloride hydrate 0.001 (g/l water).

K medium (pH 7.0)[5]: starch 25.0, soybean meal 15.0, dry yeast powder 2.0, calcium carbonate 4.0 (g/l water).

G medium[5]: starch 10.0, Polypepton 10.0, molasses 10.0, meat extr. 10.0, calcium carbonate 2.0 (g/l water).

F-1 A medium (pH 7.3): corn starch 20, glucose 5, soybean oil 5, soyaflour 50, Pharmamedia 5, Staminol 1, calcium carbonate 2 (g/l water).

N medium (pH 7.4-7.5)[5]: soybean meal 15.0, ammonium sulfate 2.0, dry yeast powder 2.0, starch 25.0, sodium chloride 5.0, calcium carbonate 4.0 (g/l water).

N' medium: glycerol 25.0, glucose 30.0, Polypepton 10.0, corn steep liquor 20.0, sodium chloride 2.0 (g/l water).

A medium (pH 7.3): malt extr. 35, corn starch 30, corn steep liquor 15, Pharmamedia 15, Sungrain 5, calcium carbonate 2 (g/l water).

H medium (pH 7.2)[5]: glucose 15.0, glycerol 10.0, soybean meal 15.0, dry yeast powder 5.0, sodium chloride 5.0, ammonium sulfate 5.0, starch 10.0, Polypepton 10.0, molasses 20.0, meat extr. 10.0, calcium carbonate 4.0 (g/l water).

P medium[5]: glucose 15.0, glycerol 10.0, Polypepton 10.0, meat extr. 10.0, calcium carbonate 4.0 (g/l water).

Nematicidal assay

The "cotton ball on fungal mat method"[1], devised by the first of the authors was used both for screening and for guiding fractionation. Samples of cultures for the assay were prepared as follows: Culture broth was prepared by inoculating 10 ml of PSM medium in a cotton plugged test tube (25×200 mm) with growth from a stock slant, and incubated with shaking at 27 ℃ for 4 days. The culture broth was separated by centrifugation at 10000 rpm for 10 min at 4 ℃ to supernatant and cells. One tenth of the supernatant was freeze-dried, dissolved in 0.1 ml of water, and injected into a cotton ball. The cotton ball after drying *in vacuo* in a desiccator was subjected to the assay.

All the cells were soaked in acetone-methanol (1:1) at room temperature for 1-3 days, and the extract was concentrated to 0.1 ml and subjected to the assay.

Another culture broth of the same strain was kept standing in a test tube at 27 ℃ for 2 weeks after the shaking mentioned above. This culture broth was also worked up in a similar way, and subjected to the assay. These are samples indicated by "shaken and subsequent stationary incubation" in Table 1.

Results and Discussion

Screening of cultures of 99 strains of fungi

The result of screening 99 strains of fungi is shown in Table 1. Growth rates of the nematode were shown only in entries which showed considerable activity in superficial appearances of the fungal mat. Strong activity (growth rate, less than 5%) was observed in entries 13 and 18, and in the statically cultured cells of entries 55 and 66. Because samples from the supernatants of the cultures of fungi 13 and 18 were dosed ten times less than those from the cells, the stronger activ-

Table 1 Nematicidal activity of cultures of 99 strains of fungi

Entry	Shaken Incubation Activity[a), G(%)[b)]		Shaken and Subsequent Stationary Incubation Activity[a), G(%)[b)]	
	Cells	Supernatant	Cells	Superntant
Fungus- 1	−	−	−	−
2	−	−	−	−
3	−	−	−	−
4	−	−	−	−
5	−	−	−	−
6	−	−	−	−
7	−	−	−	−
8	−	−	−	−
9	−	−	−	−
10	−	−	−	−
11	−	−	−	−
12	−	−	−	−
13	+, 1.1	+, 0	+, 0	+,0.5
14	−	−	−	−
15	−	−	−	−
16	−	−	−	−
17	−	−	−	−
18	+, 0	+, 0	+, 0	+, 0
19	−	−	−	−
20	−	−	−	−
21	−	−	−	−
22	−	−	−	−
23	−	−	−	−
24	−	−	−	−
25	−	−	−	−
26	−	−	−	−
27	−	−	−	−
28	−	−	−	−
29	−	−	−	−
30	−	−	−	−
31	−	−	−	−
32	−	−	−	−
33	−	−	−	−
34	−	−	−	−
35	−	−	−	−
36	−	−	−	−
37	−	−	−	−
38	−	−	−	−
39	−	−	+, 5.1	−
40	−	−	−	−
41	−	−	−	−
42	−	−	−	−
43	−	−	−	−
44	−	−	−	−
45	−	−	−	−
46	−	−	−	−
47	−	−	−	−
48	−	−	−	−
49	−	−	−	−
50	−	−	−	−
Fungus-51	−	−	−	−
52	+, 6.1	−	−	−
53	−	−	−	−
54	−	−	−	−
55	−	−	+, 2.7	−
56	−	−	−	−
57	−	−	−	−
58	−	−	−	−
59	−	−	−	−
60	−	−	−	−
61	−	−	−	−
62	−	−	−	−
63	−	−	−	−
64	−	−	−	−
65	−	−	−	−
66	+, 7.7	−	+, 1.7	−
67	−	−	−	−
68	−	−	−	−
69	−	−	−	−
70	−	−	−	−
71	−	−	−	−
72	−	−	−	−
73	−	−	−	−
74	−	−	−	−
75	−	−	−	−
76	−	−	−	−
77	−	−	−	−
78	−	−	−	−
79	−	−	−	−
80	−	−	−	−
81	−	−	−	−
82	−	−	−	−
83	−	−	−	−
84	−	−	−	−
85	−	−	−	−
86	−	−	−	−
87	+, 9.3	+, 8.9	−	−
88	−	−	−	−
89	−	−	−	−
90	−	−	−	−
91	−	−	−	−
92	−	−	−	−
93	−	−	−	−
94	−	−	−	−
95	−	−	−	−
96	−	−	−	−
97	−	−	−	−
98	−	−	−	−
99	−	−	−	−

a) observed from superficial appearances of fungal mat; +: active, −: inactive
b) growth rates calculated from nematode numbers

ity was present in the supernatants. In order to confirm that the nematicidal activity of the supernatants was not attributed to their acidity (pH 1~2), these two supernatants were assayed after adjustment of their pH values to 7. On pH adjustment to 7.1, the supernatant of entry 13 lost its entire activity, but that of entry 18 lost little activity (G % 3.41 % in the case of shaking incubation, G % 4.59 % for static incubation). The supernatant of entry 18 was, therefore, selected for further investigation.

Search for the best conditions of mass-culture of the fungus 18

In Table 2 was shown the nematicidal activity of supernatants (0.5 ml) of cultures (40 ml) of the fungus 18 in 17 kinds of media incubated in a 200 ml conical flask with shaking (170 rpm) at 27 ℃ for 4 days. The first 7 kinds of media produced more active substances. Then, the nematicidal activity of supernatants of cultures incubated in the 7 media was tested after adjustment of the pH to 7 (Table 3). Four media (PSM, Bennett's, CM, and PY) were selected for determination of incubation period. Table 4 showed that incubation of fungus 18 in PSM or Bennett's medium for 4 days produced the strongest activity.

For mass-culture, the seed culture of fungus 18 in 10 ml of PSM medium incubated in a test tube (25×200 mm) at 27 ℃ for 2 days was transferred into 150 ml of PSM or Benett's medium in a 500 ml shaking flask, and incubated at 27 ℃ on a reciprocal shaker (5 cm, 130 strokes/min) for 4, 6 and 8 days. The nematicidal activity of the supernatants of cultures incubated for 4 days was the strongest, as shown in Table 5. Therefore, fungus 18 was cultured in 10 shaking flasks, each containing 150 ml of PSM medium, for 4 days, and 1360 ml of the supernatant (pH 1.8) was obtained. The activity of 3 ml or 1.5 ml of the supernatant was shown in Table 6.

Preliminary fractionation of the supernatant obtained from mass-culture

The supernatant was extracted with ethyl acetate and then with 1-butanol. Nematicidal activity was observed only in the aqueous layer. Neither cation nor anion exchange resin (Dowex-50W, H^+ form, AG 1×8, OH^- form) retained any activity.

Charcoal column could not retain any activity

Table 2 The nematicidal activity of 0.5 ml of supernatants of cultures of fungus 18 in various media

Medium	pH[a]	Activity[b]	G(%)[c]
PY	1.2	++	0
PSM	1.6	++	0
YM	1.6	++	0
CM	1.3	++	0
Y-3	1.9	++	0
Czapek-Dox	2.1	++	0
Bennett's	1.3	++	0
No. 2	2.0	+	1.5
K	1.4	+	1.5
G	3.0	+	1.5
F	3.5	+	2.9
F1-A	2.7	+	2.9
N'	2.0	±	8.8
N	2.3	−	48.3
A	2.0	−	32.2
H	4.2	−	22.0
P	2.7	−	

a) values just after incubation
b) observed from superficial appearances of fungal mat; +: active, −: inactive
c) growth rates calculated from nematode numbers

Table 3 The nematicidal activity of pH adjusted supernatants of cultures of funguns 18

Medium	No pH Adjustment[a) 0.5 ml[b]		pH Adjustment[a] 1.0 ml[b]	
	Activity[c]	G(%)[d]	Activity[c]	G(%)[d]
PSM	++	0	++	0
Bennett's	++	0	+	2.8
CM	++	0	+	4.2
PY	++	0	++	0
Czapek-Dox	±	16.7	−	
Y-3	++	0	±	11.1
YM	++	0	±	12.5

a) after incubation
b) ml of the supernatant assayed
c) observed from superficial appearances of fungal mat; +: active, −: inactive
d) growth rates calculated from nematode numbers

Table 4 The nematicidal activity of the supernatants of cultures of fungus 18 incubated for various periods

Day	Dose[a]	Medium											
		PSM			Bennett's			CM			PY		
		pH[b]	A[c]	G(%)[d]	pH[b]	A[c]	G(%)[d]	pH[b]	A[c]	G(%)[d]	pH[b]	A[c]	G(%)[d]
4	0.50	1.3	+	0.4	0.9	++	0	2.3	−		2.4	−	
	0.25	1.3	−		0.9	+	4.6	2.3	−		2.4	−	
	1.00	7.0	+	1.7	7.0	++	0	7.0	−		7.0	+	5.2
	0.50	7.0	−		7.0	+	2.8	7.0	−		7.0	−	
6	0.50	0.8	+	0	0.8	+	0	1.9	+	2.6	3.3	+	3.9
	0.25	0.8	−		0.8	−		1.9	−		3.3	−	
	1.00	7.0	+	2.2	7.0	−		7.0	−		7.0	−	
	0.50	7.0	−		7.0	−		7.0	−		7.0	−	
8	0.50	5.3	±	27.5	2.5	+	0	3.1	−	28.6	5.1	−	
	0.25	5.3	−		2.5	±	16.0	3.1	−	23.0	5.1	−	
	1.00	7.0	−		7.0	+	0	7.0	+	0	7.0	+	3.5
	0.50	7.0	−		7.0	±	17.9	7.0	−	23.0	7.0	−	
10	0.50	6.2	−		2.7	+	0	4.3	±	28.6			
	0.25	6.2	−		2.7	−	35.7	4.3	−	55.9			
	1.00	7.0	−		7.0	+	0	7.0	+	0			
	0.50	7.0	−		7.0	−	82.1	7.0	−	38.1			
12	0.50	6.1	−		4.6	−		5.9	−				
	0.25	6.1	−		4.6	−		5.9	−				
	1.00	7.0	−		7.0	−		7.0	−				
	0.50	7.0	−		7.0	−		7.0	−				

a) ml of supernatant assayed
b) numbers other than 7.0 (the adjusted pH) are the original pH values after incubation
c) activity observed from superficial appearances of fungal mat; +: active, −: inactive
d) growth rates calculated from nematode numbers

Table 5 The mematicidal activity of the supernatants of cultures of fungus 18 incubated in quantity in 2 media for various periods

Day	Dose[a]	Medium					
		PSM			Bennett's		
		pH[b]	A[c]	G(%)[d]	pH[b]	A[c]	G(%)[d]
4	0.50	1.1	++	0	1.5	++	0
	0.25	1.1	−	7.2	1.5	±	14.5
	1.00	7.0	++	0	7.0	++	0
	0.50	7.0	−	1.8	7.0	±	9.0
6	0.50	1.6	++	0	1.9	++	0
	0.25	1.6	++	0	1.9	−	38.0
	1.00	7.0	++	0	7.0	++	1.4
	0.50	7.0	++	1.8	7.0	−	21.7
8	0.50	1.4	++	0	1.9	++	0
	0.25	1.4	+	10.8	1.9	−	57.8
	1.00	7.0	++	0	7.0	++	0
	0.50	7.0	++	0	7.0	−	

a) ml of supernatant assayed
b) numbers other than 7.0 (the adjusted pH) are the original pH values after incubation
c) activity observed from superficial appearances of fungal mat; +: active, −: inactive
d) growth rates calculated from nematode numbers

Table 6 The nematicidal activity of the supernatant of cultures of fungus 18 incubated in a large quantity in PSM medium for 4 days

Dose(mg)[a]	pH[b]	Activity[c]	G(%)[d]
5.1	0.8	++	0
2.6	0.8	++	2.9
10.3	7.0	++	0
5.1	7.0	+	3.6
2.6	7.0	−	

a) weight of the risidue of the supernatant assayed
b) numbers other than 7.0 (the adjusted pH) are the original pH values after incubation
c) activity observed from superficial appearances of fungal mat; +: active, −: inactive
d) growth rates calculated from nematode numbers

either. The chromatographic behaviors of the activity suggested that the active compound was neutral and water soluble. Further purification and structure elucidation are in progress.

References

1) Kawazu, K., Y. Nishii, K. Ishii and M. Tada : Studies on naturally occurring nematicidal substances. Pt. I. A convenient screening method for nematicidal activity. Agric. Biol. Chem., **44**(3), 631-635 (1980)
2) Kawazu, K., Y. Nishii and S. Nakajima : Studies on naturally occurring nematicidal substances. Pt. II. Two nematicidal substances from roots of *Cirsium japonicum*. Agric. Biol. Chem., **44**(4), 903-906 (1980)
3) Kawazu, K. : A novel inhibitor of pine wood nematode propagation produced by an actinomycete isolated from a wilted pine tree in Handayama, Okayama, A Report of Specified Research on Handayama. Okayama University (III), 79-82 (1989)
4) Kawazu, K., T. Murakami, Y. Ono, H. Kanzaki, A. Kobayashi, T. Mikawa and N. Yoshikawa : Isolation and characterization of two novel nematicidal depsipeptides from an imperfect fungus, strain D1084. Biosci. Biotech. Biochem., **57**(1), 98-101 (1993)
5) Ootake, N. : Koseibussitugaku. pp. 143-148, Yokendo, Tokyo (1985)
6) Kyowa Hakko, Tokyo Research Institute eds. : Biseibutu Jikken Manual, p. 259, Kodansha Scientific, Tokyo (1986)

土壌から単離した真菌の培養物についての殺線虫活性のスクリーニングと殺線虫活性のある1菌株培養上清の予備分画

河津　一儀，孫　建華，神崎　浩
（生物資源開発学講座）

土壌から単離した99菌株の真菌の培養物について，以前に著者の１人が開発した線虫増殖阻害活性を検出するバイオアッセイを用いてスクリーニングを行った．１菌株の遠心分離上清に活性を認めた．予備的に行った各種クロマトグラフィーにおける挙動から，本活性化合物は，水溶性の中性化合物であると推定した．