

菌核病菌(Sclerotinia sclerotiorum)の菌核から分離したChaetomium trilaterale var. diporum RC-5が産生する抗糸状菌物質

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The Antifungal Substance Produced by *Chaetomium* *trilaterale* var. *diporum* RC-5 Isolated from Sclerotia of *Sclerotinia sclerotiorum*

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Abstract

Chaetomium trilaterale var. *diporum* RC-5 was isolated from sclerotia of *Sclerotinia sclerotiorum* buried in soil in Okinawa Prefecture. Since the isolate inhibited mycelial growth of *S. sclerotiorum* on PDA medium, the purification of the antifungal substance was undertaken. The antifungal substance was produced in modified Richards medium. The substance was isolated by extraction with chloroform and thin layer chromatography. Its molecular formula was determined as $C_{31}H_{35}O_8N$ (mol wt 549) on the basis of elementary analysis and fast atom bombardment mass spectrometry (FAB-MS). Mycelial growth of *S. sclerotiorum* and *Botrytis cinerea* was inhibited intensively and their hyphal tips were branched abnormally by the treatment with antifungal substance. Their ED_{50} values on PDA medium were ca. 10 ng/ml, and they were more than 20 times stronger than that of cycloheximide. While, the mycelial growth of other indicators such as *Pyricularia oryzae*, *Sclerotium hydrophilum* and some isolates of *Rhizoctonia solani* was not inhibited even at 100 μ g/ml.

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Key words: *Chaetomium trilaterale* var. *diporum*, antagonism, *Sclerotinia sclerotiorum*, antifungal substance, $C_{31}H_{35}O_8N$.

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is one of the important plant pathogenic fungi which has extremely wide host range. Since this fungus forms sclerotium as survival structure which is tolerant to various adverse conditions in soil, its control is difficult. The various fungi, such as *Coniothyrium minitans*, *Trichoderma* spp., *Gliocladium* spp. and *Penicillium* spp. have been reported as the antagonist against *S. sclerotiorum*^{1).}

Previously the authors reported that ten isolates of *Chaetomium* spp. were isolated from sclerotia of *S. sclerotiorum* buried in soil in Okinawa Prefecture¹⁰⁾, and *C. trilaterale* var. *diporum* and *C. trilaterale* var. *cupreum* were identified as producer of the antifungal substance in PDA medium^{11).}

Generally, *Chaetomium* spp. have been isolated from soil and cellulose containing substrates. These fungi produce various biologically active substances, such as chetomin, chaetocin, chaetoglobosins, chaetochromins, cochliodinol, colletodiol, mollicellin, oosporein and sterigmatosystin, and those chemical structure and biological action have been reported by many researchers^{3-6, 12-15).} Cullen and Andrews⁷⁾ reported that chetomin produced by *C. globosum* showed a principal antagonism to *Venturia inaequalis*. It was also reported that *C. globosum* suppressed the development of Verticillium disease and it produced chaetoglobosin A which

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inhibited spore germination of *V. dahliae*^{2,9)}. These reports will suggest the contribution of some *Chaetomium* spp. in suppression of plant pathogen in the field because of its antibiotics productivity.

In our experiment, *C. trilaterale* var. *diporum* RC-5 isolated from sclerotia of *S. sclerotiorum* inhibited mycelial growth of *S. sclerotiorum* intensively on PDA medium. This paper reports isolation, characterization and antifungal activity of the antifungal substance.

MATERIALS AND METHODS

Producer fungus. *Chaetomium trilaterale* var. *diporum* RC-5 was used as a producer fungus. The producer was maintained on PDA or cellulose agar slants at 22°C.

Isolation of antifungal substance. The mycelial disk (7 mm in diameter) was inoculated in 100 ml modified Richards medium (20 g D-glucose, 10 g KNO₃, 5 g KH₂PO₄, 2.5 g MgSO₄·7H₂O, 20 mg FeCl₃·6H₂O, 1 mg thiamine hydrochloride, 0.05 mg biotin per 1 liter, pH 5.2) in 200 ml Erlenmeyer flasks. The cultures were conducted at 30°C for 10 days in still condition.

After removing of mycelia by filtration, the culture filtrate was adjusted to pH 3.0 with 1 N HCl, and antifungal substance was extracted with chloroform. The chloroform layer was shaken with distilled water for eliminating HCl, and then chloroform was evaporated at 30°C. The residue was dissolved in 1% NaHCO₃ and extracted neutral substances with diethyl ether. After adjusting water phase to pH 3.0, antifungal substance was reextracted with chloroform. The chloroform layer was shaken with distilled water to remove HCl, and then evaporated chloroform at 30°C. The residue was dissolved in methanol and subjected to silica gel thin layer chromatography (Wakogel B-10) by using dichloromethane-methanol-28% ammonia water (65:30:5, v/v) as the developing solvent. The active fraction was isolated from the plate, and the antifungal substance was eluted with distilled water. The eluate was adjusted to pH 3.0 with 1 N HCl and the antifungal substance was transferred to chloroform. Hexane was added to the concentrated chloroform solution to precipitate the substance.

During the course of fractionation of the antifungal substance, its activity was evaluated as follows. The mycelial disk of *S. sclerotiorum* precultured on PDA plate was placed 3 cm apart from the paper disk dipped in the test sample fraction. This plate was incubated at 22°C for 2 days, then the width of inhibition zone was measured.

Spectral analysis. Ultraviolet and visible spectra of the antifungal substance were taken with a Beckman Model DU-8 recording spectrophotometer in methanol solution. Infra-red (IR) spectrum was taken with a Jasco Model A-100 recording spectrophotometer equipped with a model BC-2 beam condenser using pellet of KBr micro disk. Fast atom bombardment (FAB) mass spectrometry was made with JEOL JMS-DX300.

Antifungal activity of isolated antifungal substance against various fungi. Indicator fungi used for antifungal activity test were listed in Table 1. The mycelial disks of the indicator fungi precultured on PDA plates were transferred on the test PDA plates (8.5 cm in diam.) containing antifungal substance at 100 and 10 µg/ml. In the case of *S. sclerotiorum* and *B. cinerea*, test plates containing 100 µg/ml to 0.1 ng/ml of the antifungal substance were also assayed to estimate ED₅₀. Cycloheximide and thiophanate-methyl were used to compare ED₅₀ with the antifungal substance. Normal PDA plates were used as the check. The test and check plates were incubated at 27°C or 22°C (for *S. sclerotiorum* and *B. cinerea*) until control culture expanded to the edge, then the hyphal length of test culture was measured.

RESULTS AND DISCUSSION

Five mg of the antifungal substance was obtained from 10 litre culture filtrate of *C. trilaterale* var. *diporum* RC-5. The precipitated antifungal substance by the addition of hexane

Table 1. Relative mycelial growth inhibition of the antifungal substance against various fungi

Indicators	Origin	Inhibition	
		at 10 µg/ml	at 100 µg/ml
<i>Alternaria bataticola</i> A4-1-2	CNAES ^{a)}	45.9 ^{k)}	51.4
<i>Fusarium oxysporum</i> f. sp. <i>melonis</i>	SU ^{b)}	47.4	52.6
<i>F. roseum</i> 78-13	KNAES ^{c)}	16.1	22.6
<i>F. solani</i> f. sp. <i>pisi</i>	HU ^{d)}	8.7	30.4
<i>Helminthosporium setariae</i> IF6625	IFO ^{e)}	28.1	68.8
<i>H. maydis</i>		63.6	81.8
<i>H. oryzae</i>		48.6	51.4
<i>Pestalotia funerea</i> Ku-7	KFTES ^{f)}	29.6	44.4
<i>Pyricularia oryzae</i> Ken53-33	NIAES ^{g)}	0.0	0.0
<i>Rhizoctonia solani</i> 71	NIAES	0.0	17.1
<i>R. solani</i> GM-8		0.0	0.0
<i>R. solani</i> C-354		0.0	27.8
<i>R. solani</i> ST-9		0.0	0.0
<i>Sclerotium delphinii</i> S2-1-2	HNAES ^{h)}	54.8	67.7
<i>S. hydrophilum</i>	NIAES	0.0	0.0
<i>Sclerotinia sclerotiorum</i> KU-1	KU ⁱ⁾	97.4	100.0
<i>S. sclerotiorum</i> KU-2	KU	100.0	100.0
<i>S. sclerotiorum</i> KU-3	KU	100.0	100.0
<i>S. sclerotiorum</i> KU-4	KU	97.5	100.0
<i>S. sclerotiorum</i> KU-5	KU	92.8	97.4
<i>Botrytis cinerea</i> 85299S		93.3	100.0
<i>B. cinerea</i> 85202R ^{j)}		93.5	100.0

a) Chugoku National Agricultural Experiment Station. b) Shinshu University.

c) Kyushu National Agricultural Experiment Station. d) Hokkaido University.

e) Institute for Fermentation, Osaka, Japan. f) Kagoshima Fruit Tree Experiment Station.

g) National Institute of Agro-Environmental Sciences. h) Hokuriku National Agricultural Experiment Station. i) Kyushu University. j) Resistant strain to thiophanate methyl. k) Percentage of inhibition.

appeared as a single yellow spot on TLC plate (Merck silica gel GF₂₅₄) at *R_f* 0.38 (Fig. 1) and it fluoresced under 366 nm of UV light.

The antifungal substance was unstable in acidic condition and turned into three reciprocally exchanging substances during the evaporation of diethyl ether extract which was extracted at pH 3.0 from the fluid. In addition, TLC solvent systems containing acetic or formic acid denatured the antifungal substance, but its antifungal activity was still remained. These results indicate that the main chemical structure required for its activity is rather stable. Its ultraviolet and visible spectrum in methanol solution showed maxima at 255, 359 (sh) and 398 nm (log *E*=4.04, 4.30 and 4.32, respectively). Its infrared spectrum was shown in Fig. 2. Since the positive and negative FAB mass spectrometry showed ions at 550 (*M*+*H*)⁺, 572 (*M*+*Na*)⁺, 588 (*M*+*K*)⁺ and 548 (*M*-*H*)⁻, respectively, its molecular weight was determined to be 549. Its molecular formula was decided as C₃₁H₃₅O₈N from elementary composition: C, 67.28; H, 6.37; N, 2.51, requires: C, 67.75; H, 6.41; N, 2.55. It was also confirmed by high resolution negative FAB-MS.

The results of antifungal activity of the isolated antifungal substance against various indicators were shown in Table 1. Mycelial growth of all isolates of *S. sclerotiorum* and *B. cinerea* was inhibited intensively. However, *Pyricularia oryzae*, *Rhizoctonia solani* GM-8, ST-9 and *Sclerotium hydrophilum* were not inhibited at 100 µg/ml. The other indicators were inhibited 8.7–63.6% of the growth at 10 µg/ml and 17.1–81.8% at 100 µg/ml. These results suggest that this antifungal substance have selective action against fungi.

ED₅₀ of the antifungal substance, thiophanate-methyl and cycloheximide against *S. sclero-*

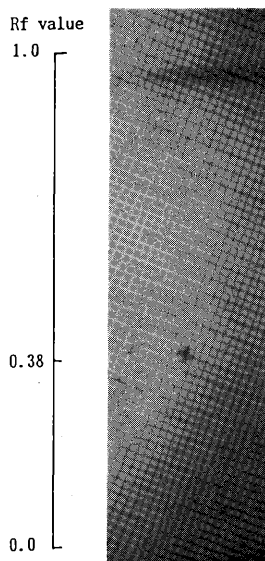


Fig. 1. Thin layer chromatogram of the antifungal substance under 254 nm UV light. Plate; Silica gel GF₂₅₄ (Merck), Solvent; dichloromethane-methanol-28% ammonia water, 65:30:5 (v/v).

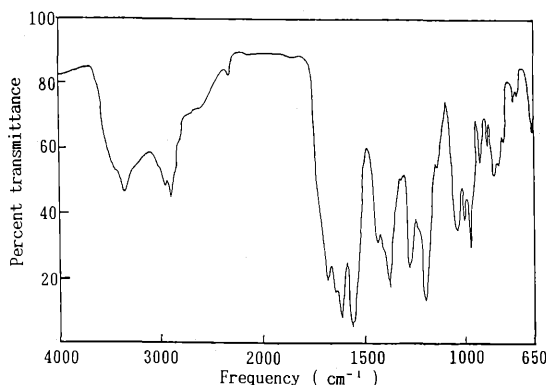


Fig. 2. Infrared spectrum of the antifungal substance in KBr micro disk.

tiorum and each isolates of *B. cinerea* were shown in Table 2. There were no differences in the ED₅₀ between thiophanate-methyl resistant and sensitive strains of *B. cinerea*. These results indicate that the antifungal substance produced by *C. trilaterale* var. *diporum* was more effective than cycloheximide and thiophanate-methyl. The hyphae on the antifungal substance containing medium were still expanded slowly even at 2 weeks later, while hyphae on cycloheximide containing medium (100 µg/ml) were not expanded at all. These results indicate that the antifungal substance has not fungicidal but fungistatic action.

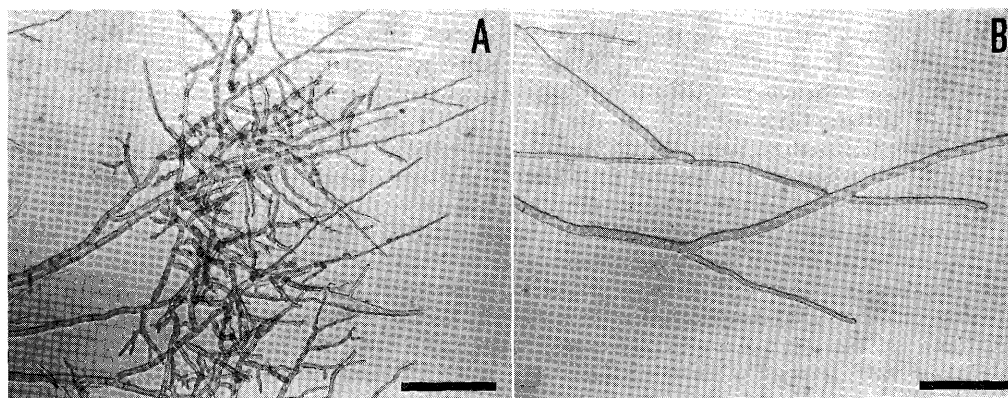
The morphological observation of growth inhibited hypha was shown in Fig. 3. The hypha of *S. sclerotiorum* was branched abnormally and the length of compartments was shorter than their check. The same abnormal branching was also observed in the case of *B. cinerea*. It has been reported that the hyphal tips of *Rhizoctonia solani* treated with the validamycins branched abnormally⁸⁾. The morphological deformations of hyphal tips of *S. sclerotiorum* and *B. cinerea* treated with the antifungal substance were similar with them. In the case of

Table 2. ED₅₀ of the antifungal substance, cycloheximide and thiophanate-methyl

Indicators	ED ₅₀ (ng/ml)		
	Antifungal substance	Cycloheximide	Thiophanate-methyl
<i>S. sclerotiorum</i> KU-1	9	200	900
<i>B. cinerea</i> 85299S	10	500	800
<i>B. cinerea</i> 85202R ^{a)}	10	400	N.D. ^{b)}

a) Resistant strain to thiophanate-methyl.

b) Not determined.

Fig. 3. Abnormal branching of hyphal tips of *Sclerotinia sclerotiorum* caused by the antifungal substance. A: Abnormal branching, B: Check. Each bar represents 200 μ m.

validamycin, the occurrence of morphological deformation was observed on nutritionally poor medium. In our experiment, however, the morphological deformation was observed on PDA medium, and not observed when *S. sclerotiorum* was grown in PD liquid medium (data were not shown). These phenomena suggest that abnormal hyphal branching is the result of secondary action of the antifungal substance.

As mentioned before, *Chaetomium* spp. produce various biologically active substances. However, the antifungal substance reported in this paper showed distinct spectral data different from that of formerly reported substances. The chemical structure of this novel substance will be the subject of future study. Since the producer of this substance was obtained from sclerotia of *S. sclerotiorum*, the substance may contribute to the suppression of *S. sclerotiorum*. The interaction between *S. sclerotiorum* and *C. trilaterale* var. *diporum* in the field, and the possibility of the antifungal substance as novel agricultural antibiotic must be investigated.

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Literature cited

1. Adams, P.B. and Ayers, W.A. (1979). Ecology of *Sclerotinia* species. *Phytopathology* 69: 896-899.
2. Anemiya, Y., Hirano, K., Iida, W., Koike, M., Kubota, T. and Kawashima, A. (1985). The effect of *Chaetomium* spp. isolated from roots of tomato against *Verticillium dahliae*. *Ann. Phytopath. Soc. Japan* 51: 327-328 (Abstr. in Japanese).
3. Brewer, D., Jerram, W.A., Meiler, D. and Taylor, A. (1970). The toxicity of cochliodinol, a metabolite of *Chaetomium* spp. *Can. J. Microbiol.* 16: 433-440.

4. Brewer, D., Jerram, W.A. and Taylor, A. (1968). The production of cochliodinol and a related metabolite by *Chaetomium* species. Can. J. Microbiol. 14: 861-866.
5. Cole, R.J. and Cox, R.H. (1981). Handbook of Toxic Fungal Metabolites: The cytochalasins. Academic Press, New York. pp. 264-343.
6. Cole, R.J., Kirksey, J.W., Cutler, H.G. and Davis, E.E. (1974). Toxic effects of oosporein from *Chaetomium trilaterale*. J. Agr. Food Chem. 22: 517-520.
7. Cullen, D. and Andrews, J.H. (1984). Evidence for the role of antibiosis in the antagonism of *Chaetomium globosum* to the apple scab pathogen, *Venturia inaequalis*. Can. J. Bot. 62: 1819-1823.
8. Iwasa, T., Higashide, E., Yamamoto, H. and Sibata, M. (1971). Studies on validamycins, new antibiotics. II. Production and biological properties of validamycins A and B. J. Antibiotics 24: 107-113.
9. Kondo, A., Amemiya, Y., Kato, T., Hirukawa, T. and Hirano, K. (1988). Antibiotic substances produced by *Chaetomium globosum*. Ann. Phytopath. Soc. Japan 54: 359 (Abstr. in Japanese).
10. Moromizato, Z., Matsuyama, N. and Tamori, M. (1987). *Chaetomium* species isolated from sclerotia of *Sclerotinia sclerotiorum* buried in soil and those antifungal activity. Ann. Phytopath. Soc. Japan 53: 415 (Abstr. in Japanese).
11. Nakashima, N., Moromizato, Z. and Matsuyama, N. (1991). Identification of antagonistic micro-organism *Chaetomium* spp. isolated from sclerotia of *Sclerotinia sclerotiorum* buried in soil. J. Fac. Agr., Kyushu Univ. 36: 109-115.
12. Powell, J.W. and Whalley, W.B. (1969). The chemistry of fungi. Part LVIII. Structure of colletodiol, a metabolite of *Chaetomium funicola*. J. Chem. Soc. (C) 911-912.
13. Udagawa, S., Muroi, T., Kurata, H., Sekita, S., Yoshihira, H., Natori, S. and Umeda, M. (1979). The production of chaetoglobosins, sterigmatocystins, O-methylsterigmatocystin, and chaetocin by *Chaetomium* spp. and related fungi. Can. J. Microbiol. 25: 170-177.
14. Sekita, S., Yoshihira, H., Natori, S., Udagawa, S., Muroi, T., Sugiyama, Y., Kurata, H. and Umeda, M. (1981). Mycotoxin production by *Chaetomium* spp. and related fungi. Can. J. Microbiol. 27: 766-772.
15. Stark, A.A., Kobbe, B., Matsuo, K., Buchi, G., Wogan, G.N. and Demain, A.L. (1978). Mollicellins: Mutagenic and antibacterial mycotoxins. Appl. Environ. Microbiol. 36: 412-420.

和 文 摘 要

中島信彦・諸見里善一・松山宣明：菌核病菌 (*Sclerotinia sclerotiorum*) の菌核から分離した *Chaetomium trilaterale* var. *diporum* RC-5 が産生する抗糸状菌物質

沖縄県の土壌中に埋めた菌核病菌 (*Sclerotinia sclerotiorum*) の菌核から分離された *Chaetomium trilaterale* var. *diporum* RC-5 が産生する抗糸状菌物質を精製し、その抗菌スペクトルを調査した。本菌を修正 Richards 培地で培養して得た培養ろ液から、クロロホルム抽出と薄層クロマトグラフィーにより分画・精製した本抗糸状菌物質は元素分析値および FAB-MS 法により $C_{31}H_{35}O_5N$ (分子量 549) と決定された。本物質は *S. sclerotiorum* と *Botrytis cinerea* の菌糸伸長を強く抑制し、菌糸先端には異常分岐が認められた。両菌に対する PDA 培地上での ED_{50} は約 10 ng/ml で、シクロヘキシミドの 20 倍以上の活性が認められた。一方、*Pyricularia oryzae*, *Sclerotium hydrophilum* と *Rhizoctonia solani* の数菌株に対しては 100 μ g/ml でもまったく抑制が観察されなかった。