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Antagonism and Structural Identification of Antifungal Compound from *Chaetomium cochliodes* against Phytopathogenic Fungi

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As a part of the integrated disease management system in greenhouse, an antifungal fungus(AF1) was isolated from greenhouse soil. It exhibited strong inhibitory activities against *Pythium ultimum*, *Phytophthora capsici*, *Rhizoctonia solani*, *Botrytis cinerea*, and *Fusarium oxysporum* based on dual culture on 1/5 strength of potato dextrose agar between antagonistic fungus and several plant pathogens. The antagonistic fungus was identified as *Chaetomium cochliodes*, based on morphological characteristics; the body of the perithecium bears straight or slightly wavy, unbranched hairs, whilst the apex bears a group of spirally coiled hairs. To investigate antagonistic principles, antifungal compound was extracted and fractionated by different solvent systems. An antifungal compound was isolated as pure crystal from its culture filtrate using organic solvent extraction and column chromatography, followed by preparative thin layer chromatography. The chemical structure of the purified antifungal compound was identified as chaetoglobosin A based on the data obtained from ^{1}H-NMR, ^{13}C-NMR, DEPT 90, 135, ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY and EI/MS. ED_{50} values of the chaetoglobosin A against *P. ultimum*, *P. capsici*, *R. solani*, *B. cinerea* and *F. oxysporum* were 1.98, 4.01, 4.16, 2.67 and 35.14 ppm, respectively.

Key words : *antagonistc fungus*, Chaetomium cochliodes, *chaetoglobosin A.*

Greenhouse production area has been rapidly increased to 40,077 ha since 1920 in Korea.[1] Development and improvement of high-tech greenhouse facilities such as frame structure, type, automation of environmental control, and crop management have geared up greenhouse horticulture to expand to one of the highest valued agricultural practices. Increased size of frame structure and investment enforced farmers to maximize productivity and to repeat monoculture. This intensive management has produced several problems relating to environment in the greenhouse such as aggravation of soil and water quality, pest control, and even health hazard for workers in closed environment. Development of integrated techniques to improve greenhouse environment for sustainable agriculture is ever demanding.

Reduction of pests can be used to minimize losses in yield and quality by integrating pest management strategies such as cultural practices, pesticides usage, and host plant resistance.[2] Biological control of diseases is one of the means to reduce the potentially harmful fungicide use. The problems of fungicide resistance of pathogens and side effects on the environment have been caused by the careless use of synthetic fungicides for control of plant disease.[3] These problems prompted researchers to undertake screening for safe compounds from microorganisms. For example, blasticidin S was thus discovered and introduced into agriculture for the control of rice blast caused by *Magnaporthe grisea*.[4] The success of blasticidin S encouraged further screening, which eventually brought about polyoxin,[5] kasugamycin,[6] validamycin,[7] and, more recently, mildiomycin.[8] These antibiotics of microbial origin have few side effects to the environment and show little toxicity to plants. They have the selective activity against plant pathogens, can be decomposed quickly after use, and do not bring about residual toxicity in soil.[9]

A large number of bioactive metabolites have been isolated from various microbial sources such as actinomycetes, bacteria, and fungi. In the present study, we isolated fungus antagonistic to five plant pathogenic fungi. An active antifungal compound was isolated and identified. Its fungicidal activity against five plant pathogenic fungi was evaluated.

Materials and Methods

Isolation and identification of antagonistic fungi. Antagonistic fungi were isolated from greenhouse soil. Appropriate serial dilutions of soil suspension in sterile H_2O were spread on rose-bengal streptomycin agar,[10] and the

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plates were incubated at 28°C for 7 days. Single colones inhibiting growth of microflora nearby were isolated and screened for antifungal activity using the petri plate assay.[11] The fungus was identified according to the morphological keys and species described by Whiteside,[12,13] especially morphology of perithecium, ascus and ascospore grown on 1/5 strength PDA [potato dextrose agar (Difco)].

Examination of antifungal spectrum of the isolated fungus. The isolated fungus was initially screened for the ability to inhibit growth of several plant pathogens on 1/5 strength of PDA plate. A culture disk (5 mm diam.) of the isolated fungus was placed at the center of a plate, and phytopathogenic fungi were placed at distance around perimeter of each plate. The plates were incubated for 1~6 days at 28°C depending on the growth rate of plant pathogens tested and examined for growth inhibition by the antagonistic fungus. The plant pathogenic fungi tested were *Pythium ultimum, Rhizoctonia solani, Phytophthora capsici, Botrytis cinerea,* and *Fusarium oxysporum.*

Isolation and purification of antifungal compound. An antifungal compound was isolated from the culture broth of the antagonistic fungus grown in a modified Czapek-dox medium(CDM), substituting asparagine for KNO_3, after growing at 28°C in a shaking incubator for 7 days. The culture broth(10*l*) was first filtered through four layers of cheese cloth, and the filtrate was then extracted with 500 m*l* of chloroform/*l* of the filtrate. The chloroform extract was combined and concentrated *in vacuo*. Approximately 3 g of crude extract was applied on a silica gel(230-400 mesh) chromatography column(20×400 mm), and eluted with chloroform-methanol step gradients. The fraction(120 mg), which exhibited biological activity, was applied on a silica gel(230-400 mesh ASTM) column(10×150 mm), and eluted with cyclohexane-ethylacetate step gradients from 1/0, 100/1, 50/1, 10/1, 1/1 to 0/1. Fifteen-milliliters of the fractions were collected. Ten-microliter aliquots from each fraction were spotted on silica gel thin layer chromatography plate(60 GF_{254}, 20×20 cm, 0.25 mm, Merck) and developed with cyclohexane-ethylacetate(1 : 1, v/v). The direct inhibition bioassay of antifungal activity on the developed TLC plate was used to identify the inhibitory compounds. A spore suspension of *B. cinerea* in 1% PDA was sprayed on top of the TLC plate developed earlier by the solvent. Inhibition zones were observed after 4-5 day incubation at 28°C in a humidified box. Fractions containing inhibitory activity were combined and dired *in vacuo*. The residues were loaded onto preparative TLC and developed with cyclohexane-methylene chloride-acetone(0.5 : 8 : 2, v/v). The antifungal compound(28mg), chaetoglobosin A, purified by preparative TLC was recrystallized in ether. Spectral data of the compound is presented in Table 1. Copies of original spectra are obtainable from the author upon request.

Identification of an antifungal compound. The ultraviolet(UV) spectrum of purified antibiotic was determined in methanol solution with Beckman DU-600 spectophotometer. Infrared(IR) spectrum was obtained with Bruker IFS 66 spectophotometer. Sample was prepared as thin films on KBr window. Proton and ^{13}C nuclear magnetic resonance spectra were obtained with JEOL JMM LA-400 spectrometer. The sample was prepared in 5 mm tube as a 25 mg/0.5 m*l* solution in CD_3OD with tetramethylsilane(Me_4Si) as an internal reference. Single-frequency, off-resonance, proton decoupled ^{13}C spectra were obtained by offsetting the decoupler 400 MHz upfield from Me_4Si in order to aid in the assignment of the carbon chemical shifts. Mass spectrum were obtained on JEOL JMS-AX505 WA spectrometer. Sample was introduced into the instrument by direct probe method. Ionization was made by electron impact at 70 eV.

Determination of antifungal activity. The serially diluted antibiotic was amended in 1/5 strength of PDA plate as 0 to 200 ppm, and a 5 mm agar plug of five phtytopathogenic fungi was placed onto the center of the plates. The diameters of mycelial growth were measured from 3 replicates of each treatment. The ED_{50} values of the mycelial growth inhibition were calculated by a probit analysis.

Determination of antifungal activity of antagonistic fungus in soil. Each pot(16×8×7cm) was filled with sterilized soil, and soil inoculum mixed with soil-borne pathogens, *P. ultimum* and *R. solani,* was added at the rate of 1% per fresh weight of pot soil. The antagonistic fungus soil inoculum was added at the rate of 1% and 5% per fresh weight of pot soil into the pots. The inoculated pots were placed in glass house for 4-5 days. After induced reciprocal action between pathogens and antagonistic fungus, six seeds of cucumber were sown in the pots. For control, cucumber seeds sown in non-treated, mixed pathogen or the antagonistic fungus were placed in the same condition. Antagonistic action of the antagonistic fungus on cucumber growth was observed. The pot soil was extracted with acetone, and its extract was fractionated with methy- lene chloride. Antifungal compound isolated from pot soil of each treatment was identified by TLC and petri-plate assay for antifungal activity.

Results

Isolation and identification of antagonistic fungus. Several antagonistic fungi isolated from the greenhouse soil were screened by the petri-plate assay against the five phytopathogenic fungi. One antagonistic fungus showing an inhibition zone was selected for identification. The fungus was identified as *Chaetomium cochliodes* based on morphological characteristics (Fig. 1). The body of the perithecium bears straight or slightly wavy, unbranched hairs, whilst the apex bears a group of spirally coiled hairs. The Chaetomiaceae easy to recognize at a glance is the perithecium of numerous long hairs. The hairs covering the upper part of the perithecium are conspicuously curly.

Antifungal spectrum of the isolated antagonistic fungus. Antifungal spectra of the isolated fungus were observed

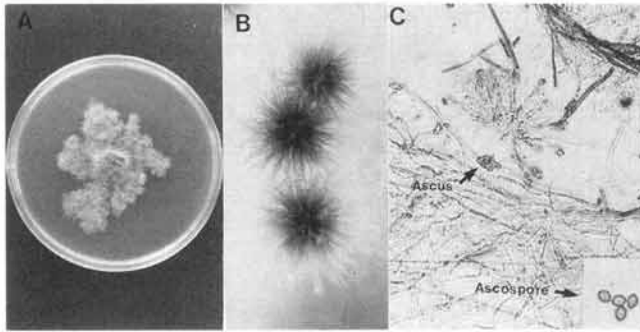

Fig. 1. Morphological characteristics of the antagonistic fungus, *Chaetomium cochliodes*. A : Colony morphology of antagonistic fungus grown on PDA. B : Phase contrast view of perithecium of numerous long hairs. C : Phase contrast view of an ascus and ascospores.

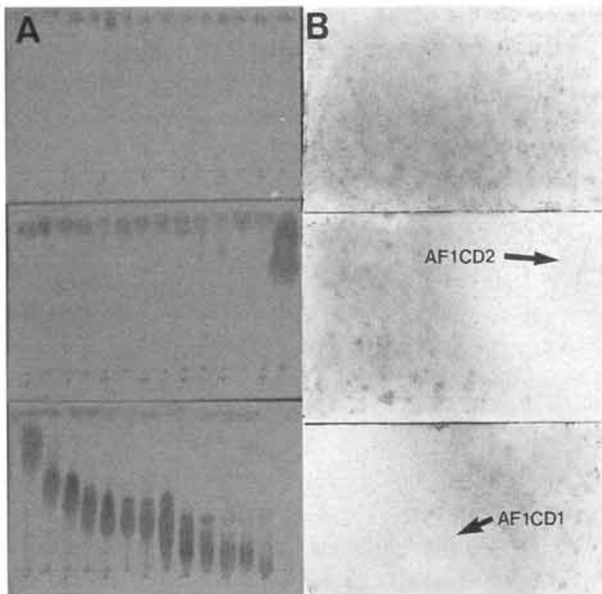

Fig. 2. Thin layer chromatogram of step gradient eluants after 2nd column chromatography. A : Each column eluant fraction was developed by TLC(cyclohexane-ethylacetate, 1 :1). B : Direct inhibition assay on a TLC plate using conidial suspension of *Botrytis cinerea*. Cleared inhibition zone area was indicated by the arrow.

on dual culture on 1/5 strength of PDA between antagonistic *C. cochliodes* and phytopathogenic fungi, *P. ultimum*, *R. solani*, *B. cinerea*, *P. capsici*, and *F. oxysporum*. It exhibited strong inhibitory activities against *B. cinerea*, *R. solani*, *P. ultimum* and *P. capsici*.

Isolation and purification of an antifungal compound. The crude chloroform extract was applied on first column chromatography. The fractions which exhibited antifungal activity were combined and applied on second column chromatography. Two separate cleared inhibition zones were observed at R_f 0.8 and 0.28(Fig. 2) by direct inhibition bioassay with *B. cinerea* on TLC developed with cyclohexane-ethylacetate(1 : 1, v/v). It was designated AF1CD1 and AF1CD2, respectively. The AF1CD1 was further

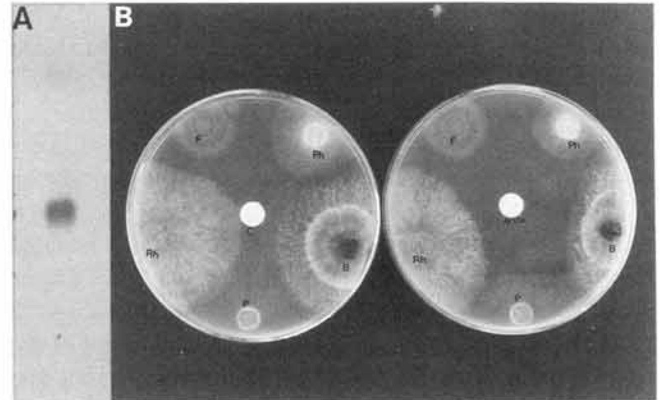

Fig. 3. The purified antifungal compound and its biological activity in a petri plate assay. A : Purified antifungal compound identified on a TLC plate. Developing solvent : $CHCl_3$-EtOAc (1 : 2, v/v), Rf : 0.38. B : Biological activity of purified antifungal compound on phytopathogenic fungi (50 μg/disc was applied : P ; *Pythium ultimum*, Ph ; *Phytophthora capsici*, Rh ; *Rhizoctonia solani*, B ; *Botrytis cinerea*, F ; *Fusarium oxysporum*). Left : Untreated control, Right : Purified antifungal compound treated.

isolated through column chromatography and preparative TLC developed with cyclohexane- methylene chloride-acetone(0.5 : 8 : 2, v/v), and pure yellow prismic crystals were obtained. The R_f value of the purified antifungal compound(AF1CD1) was 0.38 on silica gel 60 thin-layer plate developed with chloroform-ethylacetate(1 : 2, v/v), and its antifungal activity on the phytopathogenic fungi in petri-plate assay was confirmed(Fig. 3). Inhibition patterns on the pathogens by the antagonistic fungus and the purified compound were very similar, which indicates this compound may play a major role in the antagonistic activity.

Structural identification of purified antifungal compound. The compound(AF1CD1) was soluble in most organic solvents such as chloroform, ethyl acetate, acetone and methanol, but insoluble in hexane and water. Other properties are as follows: pale yellow prism from Et_2O ; mp 168 ~ 170°C. The mass spectral analysis of AF1CD1 gave a molecular ion peak(M^+) at *m/e* 528(calculated mass for $C_{32}H_{36}N_2O_5$, 528.262). Its λ_{max} (MeOH) 220, 272, 278 nm indicates the presence of an indole chromophore. The IR spectrum of AF1CD1 indicated the presence of hydroxyl or indol group and carbonyl group at 3340 and 1737cm^{-1}. The AF1CD1 gave a positive color reaction with Ehrich reagent indicating that it has indole group. The ^{1}H and ^{13}C data are shown in Table 1. The ^{13}C NMR data and DEPT experiment revealed the presence of four methyl carbons, two methylene carbons, seventeen methine carbons and nine quaternary carbons signals. Among theses signals, three carbonyl carbons (δ175 ppm~202 ppm) were identified. The ^{1}H-NMR spectrum disclosed the presence of one secondary alcohol group(δ4.96 ppm) and epoxide(δ2.74 ppm) in this compound. ^{1}H-NMR spectrum showed a pair of doublets due to an olefinic group at low filed. The 15 a/b protons were

Table 1. NMR data for antifungal compound, AF1CD1.

Position	δ_C (ppm)	δ_H (ppm)
1	175.50	(C = O)
3	54.17	3.8 (m, 1H)
4	47.87	2.8 (dd, J_1 = 5.6 Hz, J_2 = 2.2 Hz, 1H)
5	37.63	1.69 (m, 1H)
6	59.31	(Quaternary)
7	63.66	2.74 (d, J = 5.4 Hz, 1H)
8	50.14	2.09 (dd, J_1 = 10.0Hz, J_2 = 5.4 Hz, 1H)
9	65.07	(Quaternary)
10	33.60	2.8 (m, 2H)
11	13.18	1.03 (d, J = 7.3 Hz, 3H)
12	10.74	1.29 (s, 3H)
13	129.64	6.01 (m, 1H)
14	134.27	5.09 (m, 1H)
15	42.79	2.23 (m, 1H) 1.95 (m, 1H)
16	33.17	2.42 (m, 1H)
16-CH_3	21.26	0.96 (d, J = 6.6 Hz, 3H)
17	140.50	5.48 (d, J = 9.0 HZ, 1H)
18	133.70	(Quaternary)
18-CH_3	19.75	1.27 (s, 3H)
19	82.93	4.96 (s, 1H)
20	201.92	(C = O)
21	133.35	6.10 (d, J = 16.6 Hz, 1H)
22	136.06	7.23 (d, J = 16.6 Hz, 1H)
23	198.91	(C = O)
2'	125.76	6.92, (s, 1H)
3'	109.56	(Quaternary)
3'a	128.63	(Quaternary)
4'	112.61	7.23 (m, 1H)
5'	122.40	6.99 (m, 1H)
6'	120.07	6.97 (m, 1H)
7'	119.60	7.45 (m, 1H)
1'a	138.04	(Quaternary)

resonated at 1.95 and 2.23 ppm, because protons linked to the same carbon(δ42.79 ppm) from ^{13}C-^{1}H COSY experiment. In the ^{1}H-^{1}H COSY experiment, the 15 a/b protons linked to their allylic H13 resonating at δ6.01 ppm which was connected to its neighboring H8 and H14 protons resonating at δ2.09 and 5.09 ppm. The H8 proton directly linked to the H7 proton, its coupling constant value was 5.4 Hz. The H21 proton linked to H22 proton, it was existed trans form its coupling constant value, 16.6 Hz. From the above data, the antifungal compound isolated from *Chaetomium cochliodes* was identified as the known compound, chaetoglobosin A (Fig. 4).[14]

Antifungal activity of the antibiotic. This antibiotic showed strong activities on several phytopathogenic fungal species. ED_{50} values(95% limit) of the purified antifungal compound measured from degree of growth inhibition on PDA plates against *P. ultimum, P. capsici, R. solani, B. cinerea* and *F. oxysporum* were 1.98(1.48~2.62), 4.01 (3.27~5.02), 4.16(2.93~5.93), 2.67(1.81~3.81) and 35.14 (24.54~56.63) ppm, respectively. The slope and intercept of the probit regression line indicated that *P. ultimum* exhibited higher sensitivity to the increasing concentration of the antibiotic.

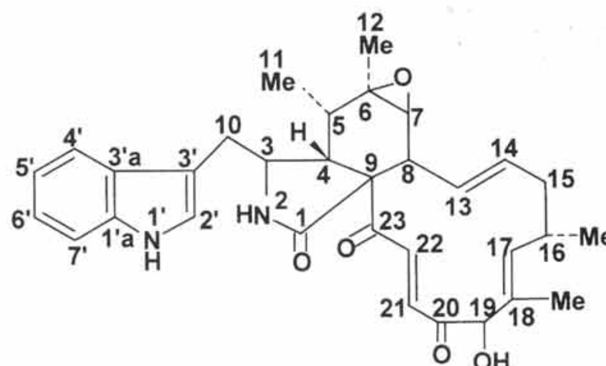

Fig. 4. Chemical structure of antifungal compound, chaetoglobosin A.

Antifungal activity of antagonistic fungus in soil. The pots treated with phytopathogenic fungal mixed inoculum, 1% per fresh weight of pot soil, exhibited 93.4% disease incidence in cucumber. But the pot treated with antagonistic fungus inoculum, 1% and 5%, per fresh weight of pot soil, gave 14% and 16.4% disease incidence in cucumber, respectively. This antagonistic fungus inhibiting various plant pathogens was effective in reducing disease incidence of cucumber seedlings caused by mixed inoculum of *R. solani* and *P. ultimum*. The antifungal compound was extracted from pot soil and compared with chaetoglobosin A standard on TLC. The pot soil treated with only *C. cochliodes* inoculum(5%) and mixed pathogen with *C. cochliodes* 5% inoculum gave the visible spot under UV light which was identical with standard compound. Its antifungal activity on *P. ultimum* in petri-plate assay was confirmed. Thus, chaetoglobosin A appeared to be the major antibiotic and could be related to the reduction of disease incidence of cucumber.

Discussion

Considerable losses are occurred due to plant diseases caused by soil-borne pathogenic fungi. In recent years, biological control of soil borne pathogens has received increasing attention as a promising supplement or alternative to chemical control. To improve efficacy of biological control, however, understanding of the mechanisms of action, nutrition, and ecology of biocontrol agents is needed. Such knowledge will lead to substantial progress in selection of superior strains, mass production, and appropriate formulation of biocontrol organisms. Many of antagonistic fungi[15] and bacteria[16] against various plant pathogens have been isolated.

C. cochliodes is antagonistic to soil-borne and seed-borne fungal pathogens, and the possibility of using them to

control plant diseases has been investigated.[17,18] In our study, the isolated antagonistic fungus from greenhouse soil by soil dilution agar method was identified as *C. cochliodes* based on morphological characteristics. It exhibited strong inhibitory activities against *B. cinerea*, *R. solani*, *P. ultimum* and *P. capsici*. Especially, *P. ultimum* was the most strongly inhibited by *C. cochliodes* among phytopathogens tested. Its antifungal compound, chaetoglobosin A, had ED_{50} value 1.98 ppm on *P. ultimum*. *C. cochliodes* can be used as a biological control agent to reduce disease development caused by *B. cinerea*, *R. solani*, *P. ultimum*, and *P. capsici* based on ED_{50} values measured if stable establishment of the antagonist in natural soil condition was made.

Production of antibiotics by *Chaetomium* species is well documented. Isolates of different *Chaetomium* spp. from soil produced several antibiotic compounds, including chaetoglobosins, in liquid culture.[19] The chaetoglobosins have been isolated from *Chaetomium globosum* Kuntze ex Fries, which were noticed by the production of toxic metabolite(s) causing polynuclear cells in cultured HeLa cells.[14] The biological functions of chaetolobosin A have been investigated.[21,22] However, there has been no report on antibiotic activities of this compound on plant pathogenic fungi even though efficacy on *Verticillium dahliae* was observed.[23]

The positive correlation between the production of chaetoglobosin A by *C. cochliodes* strains in liquid culture or soil and the efficacy in suppressing plant pathogens in the pot provides further evidence for the important of this metabolite in antagonism of *C. cochliodes* against plant pathogens.

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