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Griseofulvin from *Xylaria* sp. Strain F0010, an Endophytic Fungus of *Abies holophylla* and its Antifungal Activity Against Plant Pathogenic Fungi

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Abstract Griseofulvin has been used as an antifungal antibiotic for the treatment of mycotic diseases of humans and veterinary animals. The purpose of this work was to identify a griseofulvin-producing endophytic fungus from *Abies holophylla* and evaluate its *in vivo* antifungal activity against plant pathogenic fungi. Based on nuclear ribosomal ITS1-5.8S-ITS2 sequence analysis, the fungus was identified and labeled as *Xylaria* sp. F0010. Two antifungal substances were purified from liquid cultures of *Xylaria* sp. F0010, and their chemical identities were determined to be griseofulvin and dechlorogriseofulvin through mass and NMR spectral analyses. Compared to dechlorogriseofulvin, griseofulvin showed high *in vivo* and *in vitro* antifungal activity, and effectively controlled the development of rice blast (*Magnaporthe grisea*), rice sheath blight (*Corticium sasaki*), wheat leaf rust (*Puccinia recondita*), and barley powdery mildew (*Blumeria graminis* f. sp. *hordei*), at doses of 50 to 150 μg/ml, depending on the disease. This is the first report on the production of griseofulvin and dechlorogriseofulvin by *Xylaria* species.

Key words: Antifungal activity, endophyte, dechlorogriseofulvin, griseofulvin, plant pathogenic fungi, *Xylaria* sp.

Griseofulvin is one of the representative antifungal antibiotics and has been widely used as an antifungal drug, particularly against dermatophytes. It is a metabolic product of many species of *Penicillium* and affects the growth characteristics of various fungi. The antibiotic produces severe stunting, excessive branching, abnormal swelling, and twisting of hyphae. The antifungal activity has been demonstrated in many filamentous fungi, however, yeasts, actinomycetes, and Oomycetes are not affected.

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Chemical synthesis of griseofulvin is economically not feasible, because a number of intermediate steps are involved for the final product formation [14]. Therefore, griseofulvin is instead commercially prepared by fermentation processes than by chemical synthesis [27, 28]. The exploitation of the fermentation process plays a vital role in industrial applications and leads to techno-economic feasibility of the processes. In this aspect, the discovery of new fungal species capable of producing griseofulvin is very important. Many species of *Penicillium*, such as *P. griseofulvum*, *P. patulum*, *P. urticae*, *P. nigricans*, and *P. sclerotigenum* [17, 32], and *Aspergillus versicolor* [4] and *Streptomyces albolongus* [25] have presently been determined as typical griseofulvin-producing fungi.

In the course of our screening of antifungal endophytic fungi against six plant pathogenic fungi, an endophytic fungus isolated from the inner bark of the Manchurian fir (*Abies holophylla*) showed potent *in vivo* antifungal activities against *Magnaporthe grisea*, *Corticium sasaki*, *Botrytis cinerea*, *Puccinia recondita*, and *Blumeria graminis* f. sp. *hordei* [18]. This fungus was found to produce griseofulvin together with dechlorogriseofulvin and identified to be the genus *Xylaria* sp. (strain no. F0010), and antifungal activity of the two antibiotics was studied against plant pathogenic fungi.

Materials and Methods

DNA Extraction and PCR Amplification

The strain F0010 was plated onto potato dextrose agar (PDA; Becton and Dickinson Co., MA, U.S.A.) covered with cellophane and then incubated at 25°C for 4–5 days. Total genomic DNAs were extracted from mycelia cultured on PDA plates using AccuPrep® Genomic DNA Extraction Kit (Bioneer Corp., Taejon, Korea). From extracted genomic

DNA, the internal transcribed spacers 1, 2, and 5.8S of nuclear rDNA were amplified with ITS5 and ITS4 primers [30] using Quick PCR Premix containing *Taq* DNA polymerase, dNTPs, reaction buffer, and tracking dye (GENENMED Corp., Seoul, Korea). PCR reaction was conducted for 30 thermal cycles according to the following conditions; 1 min at 95°C for denaturation, 1 min at 52°C for primer annealing, 1 min at 72°C for extension, and 10 min at 72°C for terminal extension.

DNA Sequencing and Phylogenetic Analyses

Amplified PCR products were detected on 0.75% agarose gel through electrophoresis. Checked amplicons were purified with AccuPrep® PCR Purification Kit (Bioneer Corp.). The purified PCR products were sequenced with ABI3700 automated DNA sequencer (Applied Biosystems Inc., Foster, CA, U.S.A.). For sequencing of the ITS region, primer pairs ITS1 [30] and ITS4 were used. Sequences generated in this study were aligned with those obtained from GenBank using CLUSTAL X ver.1.83 [26] with gap opening penalty 10.0 and gap extension penalty 0.05. Using BioEdit ver.5.0.9 [10], ambiguous and uninformative variable sites were excluded and submitted to subsequent phylogenetic analyses. Phylogenetic analyses were performed based on parsimony analysis of PAUP 4.0b10 [24] [tree bisection reconnection (TBR) branch swapping, MAXTREES unrestricted, all gaps treated as missing data].

Isolation of Antifungal Substances from the F0010 Fungal Strain

Flasks containing sterile potato dextrose broth (PDB; Becton and Dickinson Co.) medium were inoculated with a culture of the strain F0010 and then incubated in the dark on a rotary shaker (150 rpm; 25°C; 14 days). After filtration, the culture filtrate (totaling 14 l) was extracted twice with equal volumes of ethyl acetate, and the organic phase was concentrated to dryness. The residue (1.5 g) was suspended in chloroform:methanol (95:5, v/v) and loaded onto a silica gel column [3.6 cm (inside diameter) by 60 cm] containing 200 g of Kiesel gel 60 (70-230 mesh; E. Merck, Darmstadt, Germany). The column was eluted with chloroform:methanol (95:5, 9:1, and 8:1, v/v), and the eluate was fractionated into three fractions called F1, F2, and F3, which were bioassayed in *in vivo* antifungal assay. The active F2 fraction (600 mg) was finally purified using preparative TLC plates (Kiesel gel 60, precoated, 0.5 mm film thickness; E. Merck) and *n*-hexane:ethyl acetate:methanol (50:50:2, v/v/v). The procedure yielded 40 mg of a less polar compound (compound 1) and 350 mg of a polar compound (compound 2) in TLC.

Spectral Measurements

To determine the chemical structures of two antifungal substances, they were analyzed using mass and nuclear magnetic resonance (NMR) spectrometry. Mass spectra were recorded on a double-focusing high-resolution mass spectrometer (JEOL JMS-DX 303; JEOL Ltd., Tokyo, Japan). NMR spectra were recorded in deuterochloroform on a Bruker AMX-500 (500 MHz) NMR spectrometer (Bruker Analytische Messtechnik Gmbh, Rheinstetten, Germany). Spectra were referenced to tetramethylsilane (TMS) (^{1}H) or solvent (^{13}C) signals.

Mycelial Growth Inhibition Assay

The two antifungal substances dissolved in dimethyl sulfoxide (DMSO) were tested for mycelial growth inhibition activity against 8 plant pathogenic fungi (*Alternaria mali*, *B. cinerea*, *Colletotrichum gloeosporioides*, *C. sasaki*, *Fusarium oxysporum*, *M. grisea*, *Phytophthora capsici*, and *Phytophthora infestans*) using the Poison Food Technique [6]. A PDA medium was used as the basal medium for all test fungi, except for *P. infestans* and *P. capsici*, for which a V-8 juice agar medium was used. Agar discs (5 mm in diameter) of test fungi were placed at the center of plates containing two antifungal substances of 200, 66.7, 22.2, 7.4, 2.5, and 0.82 μg/ml concentrations. Five replicate plates of each concentration for each fungus were incubated at 25°C for all test fungi, except for *P. infestans* and *B. cinerea*, which were incubated at 20°C. Plates containing media mixed with DMSO (1% by volume) were included as control. After incubation for 2 to 6 days, the radial growth was measured. The experiment was conducted twice and expressed as IC_{50} values (concentrations of the compound inhibiting radial growth by 50%).

***In Vivo* Antifungal Assay**

The two antifungal substances isolated from the strain F0010 were tested *in vivo* for antifungal activity against the following diseases, using the methods previously described [12, 13]: rice blast (*M. grisea*), rice sheath blight (*C. sasaki*), tomato gray mold (*B. cinerea*), tomato late blight (*P. infestans*), wheat leaf rust (*Puccinia recondita*), and barley powdery mildew (*Blumeria graminis* f. sp. *hordei*). Rice (*Oryza sativa* cv. Nakdong), tomato (*Lycopersicon esculentum* cv. Seokwang), barley (*Hordeum sativum* cv. Dongbori), and wheat (*Triticum aestivum* cv. Chokwang) plants were grown in vinyl pots (4.5 cm in diameter) in a greenhouse at 25±5°C for 1 to 4 weeks. The potted plant seedlings were sprayed with the two antibiotics dissolved in water:methanol (95:5, v/v) containing Tween 20 (250 μg/ml) as wetter and allowed to stand for 24 h. Control plants were treated with Tween 20 solution containing 5% methanol. The treated plant seedlings were inoculated with spores or mycelial suspensions of 6 plant pathogenic fungi, and disease severity was then assessed 3-7 days after inoculation. The percentage of fungal control was obtained by the following equation:

% control=100[(A-B)/A],

in which A=the area of infection (%) on leaves or stems sprayed with Tween 20 solution containing solvent alone and B=the area of infection (%) on treated leaves or sheaths. Pots were arranged to form a randomized complete block, with two replicates per treatment. The mean value (standard deviation) of the two estimates for each treatment was converted into the percentage of fungal control.

RESULTS AND DISCUSSION

Identification of the F0010 Fungal Strain

The fungal strain F0010 was identified as *Xylaria* sp., based on the nuclear ribosomal ITS1-5.8S-ITS2 sequence analysis (Fig. 1). The ITS sequence analysis revealed that the F0010 strain has more than 90% sequence similarity with *Xylaria cornu-damae* (AF163031) of GenBank.

The genus *Xylaria* is classified in the family Xylariaceae of the class of Pyrenomycetes. The Xylariaceae is a large and relatively well-known family, which is the representative of ascomycetes in most countries [29]. *Xylaria* species are common endophytes in many plants, including palms, orchids, bromeliads, aroids, ferns, and rain forest trees [1, 2, 7, 16, 19, 21, 22]. Bayman *et al.* [2] reported that *Xylaria* species was the most common genus isolated from *Casuarina equisetifolia* shoots and *Manikara bidentata* leaves, and found in 54% of *C. equisetifolia* shoots and 97% of *M. bidentata* leaves. On the other hand, many species of *Xylaria* actively decay wood of living or dead angiosperms

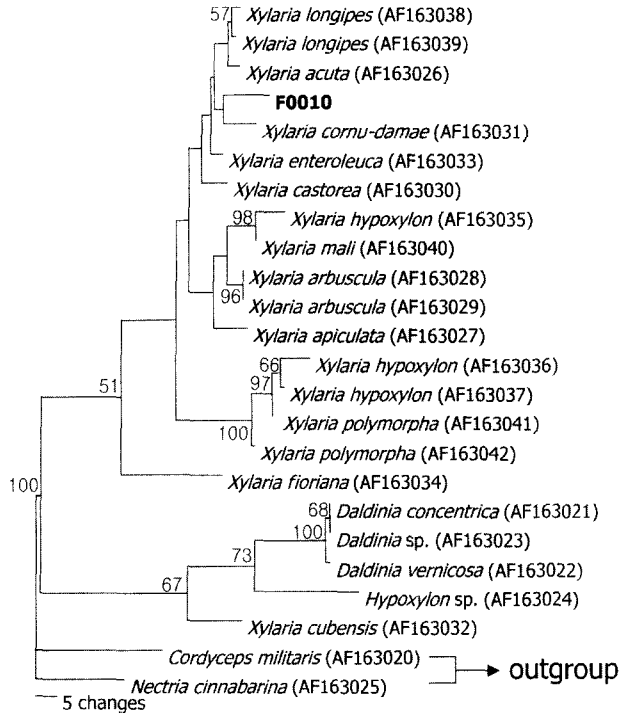


Fig. 1. Phylogenetic tree inferred from the analysis of nuclear ribosomal ITS1-5.8S-ITS2 sequences of a fungal strain F0010. This tree is one of 6 equally parsimonious unrooted trees [tree length=577 steps, CI=0.512, RI=0.660, and RC=0.337] searched by TBR option for ITS sequences. Bootstrap values were shown at nodes supported by more than 50% from 1,000 replications.

and are known to be saprobic [20]. *Xylaria* sp. F0010 was isolated from inner bark of *A. holophylla*, which is widely

Table 1. NMR data of griseofulvin and dechlorogriseofulvin isolated from *Xylaria* sp. F0010 in $CDCl_3$.

Carbon No.	Griseofulvin		Dechlorogriseofulvin	
	$^{13}C^a$	^1H (multi, J)^b	^{13}C	^1H (multi, J)
2	90.7		89.9	
3	192.5		192.5	
3a	105.1		104.7	
4	157.7		159.1	
4-OCH_3	57.0	4.04 (s)	57.0	3.63 (s)
5	89.4	6.14 (s)	88.5	6.24 (d, 1.8)
6	164.6		171.3	
6-OCH_3	56.4	4.04 (s)	56.1	3.90 (s)
7	97.2		93.3	6.05 (d, 1.7)
7a	169.5		176.0	
1'	170.8		170.3	
1'-OCH_3	56.7	3.63 (s)	56.6	3.91 (s)
2'	104.8	5.55 (s)	104.3	5.55 (s)
3'	197.1		197.4	
4'	40.0	2.44 (dd, 16.7, 4.6) 3.04 (dd, 16.0, 3.5)	40.0	2.41 (dd, 16.8, 4.7) 3.05 (dd, 16.8, 13.4)
5'	36.4	2.86 (m)	36.5	2.75 (m)
5'-CH_3	14.2	0.97 (d, 6.8)	14.2	0.97 (d, 6.7)

[a]125 MHz.
[b]500 MHz

distributed in Korea. Therefore, *Xylaria* species are supposed to be common endophytes in woody plants in Korea. The active isolate has been deposited at SNU Fungus Culture Collection (SFCC), School of Biological Sciences, Seoul National University, Korea.

Characterization of Antifungal Substances Produced by *Xylaria* sp. F0010

The liquid culture of *Xylaria* sp. F0010 exhibited potent and broad antifungal activity against plant pathogenic fungi [18]. From the liquid cultures of *Xylaria* sp. F0010, two antifungal substances were purified: compound 1 and compound 2. The low-resolution (LR)-electron impact mass spectra indicated that the molecular weights of compound 1 and compound 2 were 318 and 352 daltons, respectively. The ^{1}H- and ^{13}C-NMR spectra and the ^{1}H-^{13}C COSY spectra of the two compounds were also obtained. The connectivity of proton and carbon atoms is presented in Table 1. By compiling all the mass and NMR spectral data, the compound 1 and compound 2 were finally identified as dechlorogriseofulvin and griseofulvin, as previously reported by Grove *et al.* [9].

Griseofulvin is produced by many species of *Penicillium*, such as *P. griseofulvum*, *P. patulum*, *P. urticae*, *P. nigricans*, and *P. sclerotigenum* [17, 32]. It is also produced by *A. versicolor* [4] and *S. albolongus* [25]. *Xylaria* species are known to produce various secondary metabolites, such as multiplolides A and B [3], xyloketals A, B, C, D, and E [15], depudecin, phaseolinone, phomenone, 19,20-epoxycytochalasin Q, (E)-methyl-3-(4-methoxyphenoxy)propeate [11], and xylarenals A and B [23]. To the best of our knowledge, this is the first report on the production of griseofulvin by *Xylaria* species.

Antifungal Activity of Dechlorogriseofulvin and Griseofulvin

Griseofulvin isolated from *Xylaria* sp. F0010 inhibited the mycelial growth of some of the tested plant pathogenic fungi (Table 2). Fungal sensitivity varied, however, according to the fungal species. *C. gloeosporioides* was the most sensitive, and the other fungi - except for *P. infestans* and *P. capsici*, which belong to Oomycetes - were relatively less sensitive to the compounds with lower values of IC_{50} than 30 μg/ml. The mycelial growth of *P. infestans* and *P. capsici* were hardly inhibited even at 200 μg/ml. Compared to griseofulvin, dechlorogriseofulvin showed much weaker antifungal activity against all fungi tested.

Table 2. Inhibitory action of griseofulvin and dechlorogriseofulvin, isolated from *Xylaria* sp. F0010, against the mycelial growth of plant pathogenic fungi *in vitro*.

Fungal species	IC_{50} (μg/ml)[a]	
	Griseofulvin	Dechlorogriseofulvin
Alternaria mali	18	>200
Botrytis cinerea	5.0	>200
Colletotrichum gloeosporioides	1.7	>200
Corticium sasaki	11	>200
Fusarium oxysporum	30	>200
Magnaporthe grisea	1.7	>200
Phytophthora capsici	>200	>200
Phytophthora infestans	>200	>200

[a]Concentration required to inhibit the growth of fungi 50%.

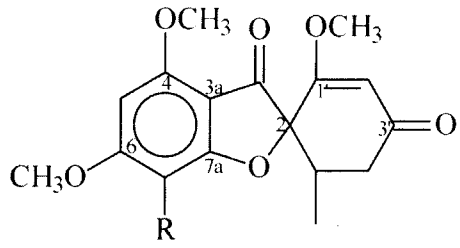
The *in vivo* antifungal activities of griseofulvin and dechlorogriseofulvin are given in Table 3. Griseofulvin effectively inhibited the development of rice blast, rice sheath blight, wheat leaf rust, and barley powdery mildew among the six plant diseases tested. It was also active *in vivo* against *B. cinerea* on tomato plants. The compound, however, was virtually inactive against *P. infestans in vivo* at 150 μg/ml. On the other hand, dechlorogriseofulvin inhibited only the development of rice sheath blight and barley powdery mildew, but hardly controlled the development of the other plant diseases. The *in vivo* antifungal spectrum of griseofulvin was similar to that of the liquid broth of *Xylaria* sp. F0010, from which the two antifungal substances

Table 3. *In vivo* antifungal activity of griseofulvin and dechlorogriseofulvin, isolated from *Xylaria* sp. F0010 from *Abeis holophylla*, against various fungal pathogens[a].

Fungal species	Host	Control value (%)[b]			
		Griseofulvin		Dechlorogriseofulvin	
		150 (μg/ml)	50 (μg/ml)	150 (μg/ml)	50 (μg/ml)
Magnaporthe grisea	Rice	95±1.9	65±8.8	8±2	0
Corticium sasaki	Rice	100	100	70±10	30±10
Botrytis cinerea	Tomato	60±2.9	10±6.0	25±4.4	33±1.0
Phytophthora infestans	Tomato	0	0	0	0
Puccinia recondite	Wheat	90±3.3	87±1.9	0	0
Blumeria graminis f. sp. *hordei*	Barley	90±3.3	90±3.3	93±0	50±17

[a]The plant seedlings were incubated with spores or mycelial suspensions of the test organisms 1 day after the chemical solutions were sprayed to run off on the leaves.

[b]Each value represents mean of three replicates±standard deviation.



Griseofulvin: R = Cl
Dechlorogriseofulvin: R = H

Fig. 2. Chemical structures of griseofulvin and dechlorogriseofulvin.

were isolated, thus suggesting that griseofulvin was the main component of the antifungal activity of the liquid broth of *Xylaria* sp. F0010.

Griseofulvin was used to control early blighting of tomatoes and the *Botrytis* disease of lettuce in Japan [31]. The present study indicated that griseofulvin controlled more effectively the development of rice blast, rice sheath blight, wheat leaf rust, and barley powdery mildew than the *Botrytis* disease, tomato gray mold. At present, this is the first report of griseofulvin on potent *in vivo* antifungal activity against rice blast, rice sheath blight, wheat leaf rust, and barley powdery mildew.

Griseofulvin has been shown to cause distortion of *Botrytis alli* and other fungi [31]. This antibiotic causes severe stunting, excessive branching, and abnormal swelling and twisting of hyphae. The chemical is fungistatic *in vitro* for various species of filamentous fungi except for Oomycetes. In this study, griseofulvin was found not to be active against *P. infestans* and *P. capsici* both *in vitro* and *in vivo*. Since the evolutionary history of Oomycetes is different from that of the so-called "higher fungi," such as Ascomycotina, Deuteromycotina, and Basidiomycotina, they are generally insensitive to most of the broad-spectrum fungicides that are currently available. Because most Oomycetes lack chitin, they are insensitive to antifungal agents targeted with chitin synthetase; namely, the polyoxins and nikkomycins [8]. They are also resistant to the benzimidazole fungicides, which specifically inhibit microtubule polymerization, indicating that the structure of Oomycetes tubulin is different from that of other fungi [5]. Griseofulvin is thought to interfere with the microtubule system by disrupting the microtubule spindle structure. Thus, insensitivity of griseofulvin against Oomycetes may be due to modification of the biochemical target site or lack of uptake.

In this study, we reported for the first time the production of griseofulvin by *Xylaria* species. Griseofulvin has been used for the past 40 years in the treatment of dermatophyte infections. In addition, it has been used in the treatment of malignant and inflammatory diseases. Although chemical synthesis of griseofulvin was reported by Lednicer and Mitscher [14], it is economically not feasible, because a number of intermediate steps are involved. Therefore, the drug is commercially prepared by fermentation of *Penicillium* species. Griseofulvin was produced with a maximum rate of 2.96 g/l after 13 days of incubation in shake culture containing a corn steep liquor-lactose medium by a mutant strain of *P. patulum* (Aytoun and Mcwilliam, 1957. Mutants of the genus *Penicillium*. Brit. Pat. 788,118). *Xylaria* sp. F0010 in the present study produced 0.8 g/l of griseofulvin and 0.5 g of dechlorogriseofulvin after 15 days of incubation in shake culture containing a yeast extract-polypeptone-glucose medium (data not shown). The productivity of griseofulvin by *Xylaria* sp. F0010 may be increased through optimization of fermentation processes and development of high yielding mutant strains. Thus, *Xylaria* sp. F0010 is expected as a new fungal strain available for the bulk production of griseofulvin.

Control of a number of plant diseases under commercial conditions has relied mainly on the application of a high number of fungicide sprays per season. Repeated application of some fungicides has caused residual toxicity, environmental pollution, phytotoxicity, and increase of resistant populations. For these reasons, the search for alternative control measures, such as biological control agents and plant extracts, has been challenging. Liquid cultures of *Xylaria* sp. F0010 and griseofulvin exhibited potent broad-spectrum antifungal activity against rice blast, rice sheath blight, tomato gray mold, wheat leaf rust, and barley powdery mildew. *Xylaria* sp. F0010 apparently has potential as a biological control agent for the control of various plant diseases, except for several plant diseases caused by Oomycetes. Further studies on the development of microbial fungicide using *Xylaria* sp. F0010 are in progress.

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