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Identification of *Aspergillus* Strain with Antifungal Activity Against *Phytophthora* Species

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Abstract Fungal strain CGF was isolated from the soil of ChungNam Province, South Korea. Based on the 28S rDNA sequence analysis and the sequence of the internal transcribed spacer (ITS) region of ribosomal DNA, together with morphological and cultural characteristics, this strain was identified as *Aspergillus sclerotiorum* and renamed *Aspergillus sclerotiorum* CGF. This is the first strain of *Aspergillus sclerotiorum* identified in Korea. When the antifungal activity of *A. sclerotiorum* CGF was evaluated, among the phytopathogenic fungi, mycelial growth of only *Phytophthora* species was inhibited. Germination of *P. capsici* zoospore was also inhibited. The bioactive compound of *A. sclerotiorum* CGF was highly thermo- and pH-stable.

Key words: Antifungal agent, *Aspergillus sclerotiorum*, internal transcribed spacer (ITS), *Phytophthora* disease

Species of *Phytophthora* cause a variety of diseases on many different types of plants, ranging from seedlings of annual vegetables or ornamentals to fully developed fruit and forest trees. *Phytophthora* disease caused by *Phytophthora* species is one of the most devastating soilborne diseases of economic crops. Most species of *Phytophthora* cause root rots, damping-off seedlings, and rots of lower stems, tubers, and corms. Others cause rots of buds or fruits and some cause blights of the foliage, young twigs, and fruit [1].

Since the cause of late blight of potatoes and tomatoes was named *Phytophthora infestans* (Mont.) de Bary in 1876, 59 species and 5 varieties have been reported worldwide. The damage to cocoa crops caused by *Phytophthora* disease alone results in worldwide annual losses of over 2 billion dollars. It is estimated that the economic loss caused by *Phytophthora* diseases in USA amounts to a few billion dollars [4]. In Korea, 21 species of *Phytophthora* have been recorded to date. Considering the local distribution and the incidence of the genus *Phytophthora* and the extent of loss, *P. cactorum*, *P. capsici*, *P. drechsleri*, *P. infestans*, and *P. nicotianae* have been reported as the major causal agents. Specifically, *P. nicotianae* and *P. drechsleri*, which occur on 22 and 21 crops, respectively, have been shown to be the pathogens having the widest range of hosts [7]. The crops that suffer serious losses are mainly solanaceous plants and cucurbits. Also, *Phytophthora* diseases are a serious problem for various kinds of fruit trees and flowering and potted plants [6, 9, 11, 14]. About 70 million dollars are lost due to diseases of pepper, which represents approximately 20% of the area for vegetables cultivation in Korea [7].

Chemical controls have been used to eradicate *Phytophthora* disease, and some chemicals are used to protect plant tissues from infection or to inhibit further growth of the fungi in plant tissues. Although fungicides play an important role in crop protection, intensive use can have adverse effects on crops, useful antagonistic organisms, and predators or parasites of noxious insects. However, the most troublesome serious problem is the development of fungicide-resistant strains, leading to failures of disease control: similar to human pathogenic microorganisms, phytopathogens are also prone to developing 'drug' resistances that substantially decrease the effectiveness of these synthetic chemicals [3, 10].

Current concerns about the side effects of chemical agents as environmental pollutants in agricultural practices have resulted in the search for safer antifungal agents for biological controls [8, 13, 18, 19]. Efforts have been focused on secondary metabolites of microorganisms for potentially useful products as commercial fungicides or as lead compounds [5, 12, 20].

In the course of screening for new antifungal agents, fungal strain CGF was found to produce an antibiotic that was active against *Phytophthora* species. Based on the analysis of its rDNA sequence, and morphological and cultural

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characteristics, this strain was identified as *Aspergillus sclerotiorum*. In this paper, we report the taxonomy of the producing strain, its fermentation, and antifungal activities.

MATERIALS AND METHODS

Fungal Strains

The microorganism, fungal strain CGF, was isolated from soil sample in ChungNam Province, Korea. *Aspergillus bridgeri* NRRL 13000, *A. fresenii* NRRL 6161, *A. sclerotiorum* NRRL 4901, and *A. sclerotiorum* NRRL 5584 were kindly supplied by Dr. Stephen W. Peterson (Curator of the *Aspergillus* collection) of the Agricultural Research Service Culture Collection in U.S.A. *Aspergillus sclerotiorum* NRRL 415 was kindly supplied by the Korean Collection for Type Cultures (KCTC). The phytopathogenic fungi, *Aspergillus flavus* KACC 40250, *Botrytis cinerea* KACC 40573, *Fusarium oxysporum* KACC 40053, *F. solani* KACC 40384, *Phytophthora infestans* KACC 40718, *Pythium ultimum* KACC40705, *Rhizoctonia solani* AG-1 KACC 40101, *Sclerotium rolfsii* KACC 40832, and *Trichoderma harzianum* KACC 40784, were kindly supplied by the Korean Agricultural Culture Collection (KACC). *Phytophthora drechsleri*, *P. capsici*, *P. cactorum*, *P. cambivora*, and *P. nicotianae* in our laboratory stock were used as the test microorganisms for bioassay.

Preparation of Genomic DNA

Conidia from cultures on PDA slants were used to inoculate 50 ml of PDB in 250-ml Erlenmeyer flasks. Flasks were incubated on a rotary shaker at 25°C and 200 rpm. Mycelium was harvested by filtration from the flasks after 2–3 days of growth and washed several times with saline solution (0.8% NaCl and 0.0125% Tween 20). Mycelium suspended in saline solution was pelleted by centrifugation for 5 min in a bench-top microcentrifuge (5,000 rpm). The pellet was resuspended in 500 µl lysis buffer [50 mmol l^{-1} EDTA (pH 8.0) and 0.3% SDS], and the tube was left for 10 min. All of the content was transferred to a sterilized fresh tube containing glass bead (1.2 mm). The samples were vigorously vortexed for 1 or 2 min and centrifuged at 14,000 ×*g* for 3 min. The supernatant was added to two volumes of isopropanol, gently mixed, and spun down at 14,000 ×*g* for 10 min. The pellet was then suspended in ultra pure water. These DNA preparations were used for DNA amplification.

Amplification and Cloning of rDNA

The 28S rDNA was amplified, using the universal primers CTB6 (5'-GCATATCAATAAGCGGAGG-3') and TW13 (5'-GGTCCGTGTTTCAAGACG-3'). The ITS 1-5.8S-ITS 2 region was amplified, using the universal primers ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3'). ITS 1 is at the 3' end of the 18S rDNA gene and ITS 4 is at the 5' end of the 28S rDNA gene. One hundred µl of PCR reaction mixture contained 2 µl of genomic DNA, 4 µl each of primers CTB6 and TW13 or ITS 1 and ITS 4, 10 µl of 10× Tag buffer (Takara, Japan), 4 µl each of dNTP (2.5 mmol l^{-1}), 1 µl of Taq DNA polymerase (5 U, Takara, Japan) and 75 µl of distilled water (D.W.). Amplification was performed in a Minicycler (M. J. Research, Watertown, U.S.A.). Thermal profiles were 94°C, 60 sec (denaturation); 60°C, 90 sec (annealing); 72°C, 60 sec (extension), for a total of 30 cycles. According to the protocol for ligations, using the pGEM-T Easy Vector and 2X Rapid Ligation Buffer (A1360, Promega, U.S.A.), a recombinant DNA molecule was constructed. The ligation reaction (10 µl), containing 3 µl of PCR product, 1 µl of T4 DNA ligase, 1 µl of pGEM-T Easy Vector, and 5 µl of 2X Rapid Ligation Buffer, was incubated for 60 min at room temperature. By adding 10 µl of ligation mixture to 200 µl of competent cell, recombinant DNA molecule was transformed in the host cell (DH5α, EndA$^-$ strain of *E. coli*). Plasmid DNA was purified, using a Wizard Plus SV miniprep DNA purification system (A1130, Promega, U.S.A.).

Sequencing and Data Analysis

Direct sequencing of the fragments was performed on an Automated DNA Sequencer (ABI3700, Applied Biosystems Inc., U.S.A.). Sequences were determined from both strands, using CTB6 and TW13 or ITS 1 and ITS 4 as primers. Sequences were aligned using Clustal X software (Version 1.8). The search for sequence identity in the GenBank DNA Database was performed with Advanced Blast Search. Phylogenetic analysis was performed with the Clustal X program using neighbor-joining analysis [21].

Cultural Characteristics

Spore suspensions were prepared by taking a loop of spores from a slant and mixing them in Eppendorf tubes containing 0.5 ml of a sterile mixture of 0.2% agar and 0.05% Tween 80. Plates containing 25 ml of each medium were equidistantly inoculated with the spore suspensions at three points using a needle. The media used were as follows: Czapek yeast extract agar with trace metals (CYA), Czapek yeast extract agar with 20% sucrose (CY20S), Czapek agar (CZ), malt extract agar (MEA), potato dextrose agar (PDA), corn meal agar (CMA), Oat meal agar (OMA), Sabouraud dextrose agar (SDA), Yeast extract-malt extract agar (YMEA), and MY20 [15]. Morphological properties were examined at 25°C after 7 days on various media. For microscopic observations, the medium used was MEA, and slide glass culture was carried out at 28°C for 3 days.

Fermentation

Aspergillus sclerotiorum CGF was cultured on potato dextrose agar (PDA, potato infusion 4 g; dextrose 20 g;

agar 15 g; distilled water 1 l, Difco) plate at 28°C. One hundred ml of potato dextrose broth (PDB, potato starch 4 g; dextrose 20 g; distilled water 1 l, Difco) in 500-ml Erlenmeyer flask was inoculated with 10 agar pieces (5×5 mm) from the 12-day cultured plates and incubated at 28°C for 11 days in the shaking incubator with 200 rpm. The culture filtrate was obtained by using GF/C filter (Whatman) from the culture broth.

Antifungal Activity Against Phytopathogenic Fungi

The antifungal activity against plant pathogenic fungi was examined on three media: PDA, malt extract agar (MEA, malt extract 20 g; peptone 1 g; glucose 20 g; agar 20 g; distilled water 1 l), and V10 juice agar (V8 juice 100 ml; $CaCO_3$ 1 g; agar 17 g; distilled water 900 ml). Thus, the spore suspension of *A. flavus* (1×10^7 spore ml^{-1}) was added to molten PDA and then 5 ml of seeded agar was overlaid on the PDA plate. After hardening, the sterile paper discs soaked with culture filtrate (50 µl) of *A. sclerotiorum* CGF were placed on the center of the overlaid plates, and the diameter of the inhibition zone was then measured. The agar pieces of *B. cinerea*, *F. oxysporum*, *F. solani*, *P. ultimum*, and *R. solani* cultured on the PDA plates, *S. rolfsii* and *T. harzianum* cultured on the MEA plates, and *P. infestans* cultured on the V10 juice agar plate were prepared by using a cork borer (#3, 6 mm in diameter). The agar pieces were placed at 3 cm away from the paper disc soaked with culture filtrate (50 µl) of *A. sclerotiorum* CGF on PDA, MEA, and V10 juice agar plates, respectively. The distances of inhibition zone were observed after 5–7 days of incubation.

Bioassay for Antifungal Activity Against *Phytophthora* Species

Antifungal activity was measured by the paper disc diffusion method. *Phytophthora* species were cultured on oatmeal agar (Difco, U.S.A.) at 25°C for 7 days. Five agar pieces (0.5×0.5 cm) of fungi were inoculated in PDB and incubated at 25°C for 5 days on a rotary shaker at 150 rpm. Culture broth was homogenized at 10,000 rpm at room temperature for 2 min, and 5 ml of homogenized broth were added to 100 ml of top potato dextrose agar and 5 ml of seeded agar was then overlaid on the PDA plate. Fifty µl of culture supernatants were loaded on paper discs (8 mm, thick, Advantec, Japan). The paper discs were air dried, and then transferred onto the surface of the top agar overlay containing each organism. Those culture supernatants with antifungal activity showed clear zones around the paper discs after 5 days of incubation at 25°C.

pH and Thermal Stabilities of Antifungal Agent

The pH of 10 ml of culture filtrate was adjusted to the 2–11 range and stored in a freezer. After 24 h, all samples were neutralized and bioassayed against *P. drechsleri*. For thermal stability assay, culture filtrate was kept in a water bath at 80°C, and 5 ml of the sample were taken out at 0.5, 1, 2, 4, 6 h and then bioassayed against *P. drechsleri*.

RESULTS AND DISCUSSION

The D1/D2 region of the 28S rDNA sequence of fungal strain CGF is comprised of 577 bases. The sequence of fungal strain CGF was deposited in the GenBank under accession no. AY338959. The 577 bp sequence was aligned with all the presently available 28S rDNA gene sequences in the GenBank databases. The phylogenetic analysis of fungal strain CGF, using its 28S rDNA sequence data, suggested that fungal strain CGF is closely related to *A. fresenii* NRRL 6161, *A. sclerotiorum* NRRL 5584, *A. sclerotiorum* NRRL 4901, and *A. bridgeri* NRRL 13000 [16]. As shown in Table 1, comparison of the 28S rDNA sequence of fungal strain CGF and these four strains showed only two nucleotides sequence difference with an A in position 166 and a T in position 466. The ITS1-5.8S-ITS2 region of fungal strain CGF and the above-mentioned strains was analyzed and deposited in the GenBank (Table 1). The ITS1-ITS2 region of fungal strain CGF was aligned to construct a phylogenetic tree (Fig. 1). BLAST analysis data on the sequences of the ITS1-ITS2 of fungus CGF showed identity with *A. sclerotiorum* NRRL 4901. It also showed very close resemblance with *A. sclerotiorum* NRRL 5584

Table 1. *Aspergillus* strains and rDNA sequence determined in this study.

Strain	Strain number and origin		28S rDNA sequence			ITS1-5.8S-ITS2 sequence
			Position		GenBank accession no.	GenBank accession no.
			166	466		
A. sclerotiorum	CGF	Soil, Korea	A	C	AY338959	AY338960
A. fresenii	NRRL 6161	Nut, Canada	G	T	AF433125*	AY338961
A. sclerotiorum	NRRL 5584	Soil, India	G	C	AF433121*	AY338962
A. sclerotiorum	NRRL 4901	Air, Israel	G	C	AF433110*	AY338963
A. bridgeri	NRRL 13000	Soil, U.S.A.	G	C	AF433044*	AY338964

*Peterson [16].

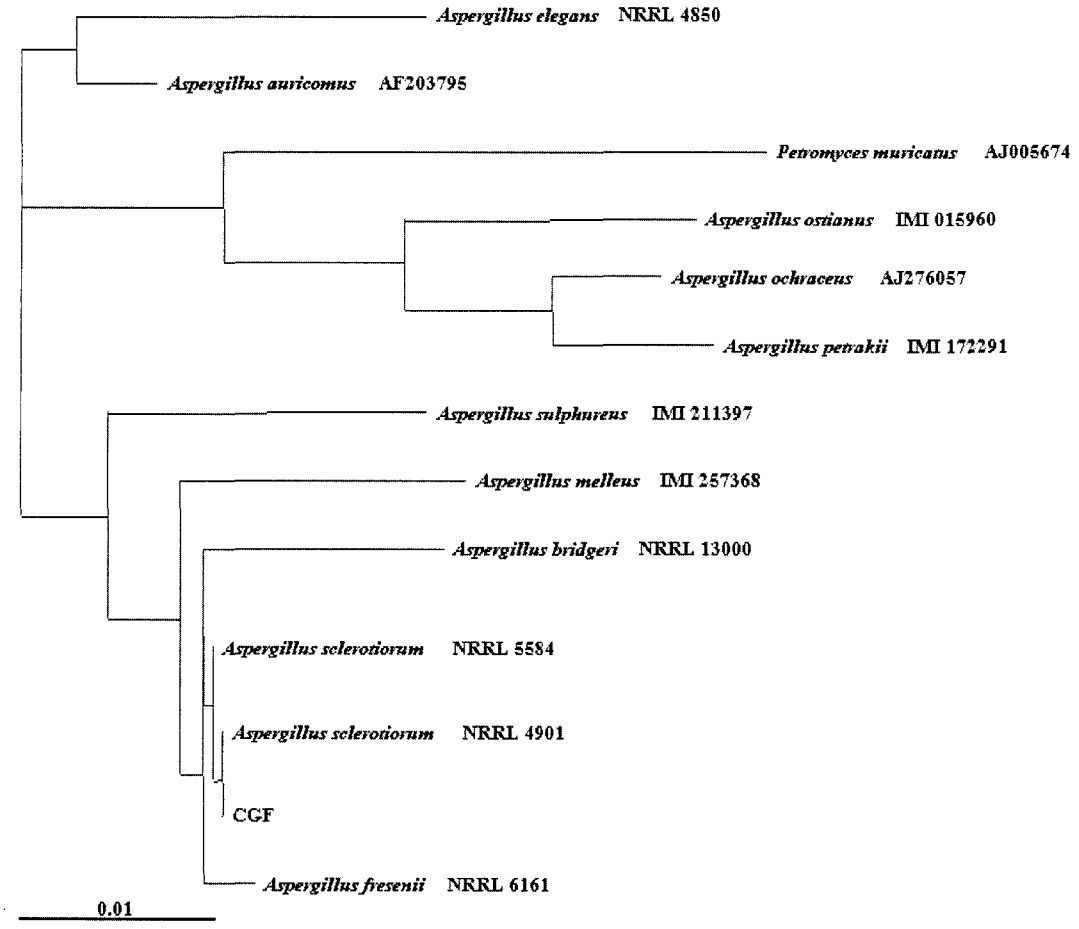

Fig. 1. Phylogenetic tree based on ITS1-ITS2 sequences. The scale bar indicates 0.01 substitutions per nucleotide.

(99.7% similarity), *A. fresenii* NRRL 6161 (99.3% similarity), *A. sulphureus* IMI 211397 (97.8% similarity), *A. melleus* IMI 257368 (97.8% similarity), and *A. sclerotiorum* AF203801 (96.8%) [22]. Strain CGF had an ITS1-5.8S-ITS2 sequence identical with that of *Aspergillus sclerotiorum* NRRL 4901. Therefore, this strain was tentatively identified as *Aspergillus sclerotiorum* CGF.

Cultural characteristics of *A. sclerotiorum* CGF on various media are summarized in Table 2. *A. sclerotiorum* CGF grew well on various culture media, attaining 2.3-6.2 cm diameter after 1 week and over 4.8 cm diameter after 2 weeks at 25°C. When CGF was incubated at 37°C, it grew restrictedly on all agar media, except for CY20S and MY20. When *A. sclerotiorum* CGF was incubated on CZ (37°C) and MEA (25°C) for 10-14 days, colony diameter was 60-75 mm and 13-18 mm, respectively. These results are quite similar to those reported by others [2, 17, 22]. This organism formed creamy-yellow to yellow-brown colonies, and the reverse color was pale-yellow to yellow-brown. The colony surface was circular with an entire planar and sometimes raised, and sulcate. Soluble pigment was absent on the media tested. Sclerotia were formed on all media, except for CMA. Okuda *et al.* [15] reported that a variety of factors such as medium ingredient, types of Petri dishes, volume of medium, method of inoculation, and incubation conditions affect the cultural characteristics of *Penicillium* and *Aspergillus*.

Figure 2 shows a microphotograph of *A. sclerotiorum* CGF, showing conidial heads and conidiophore. Conidiophore stipes were smooth or roughened, light yellow, and 250-450×5-7 μm long. Conidiophores were a little abundant on most media, except for CMA. The conidia were smooth, globose, and typically measured 2.5 μm in diameter. Conidial heads were radiate heads, splitting in columns. Vesicles were globose and 17-25 μm in diameter. Phialides were borne on metulae, 5-7×2.5-3 μm. Metulae were 5-6×2-2.5 μm. Sclerotia were globose to subglobose, creamy white to pale yellow, and 0.5-1.3 mm. The diameter of the vesicle and the lengths of conidiophore and sterigmata were somewhat smaller than those reported by some researchers, possibly due to the short cultivation time (3 days) [2, 17, 22].

A. sclerotiorum CGF, NRRL 415 (type strain) and NRRL 4901 were compared by colony diameter and cultural

Table 2. Cultural characteristics of *A. sclerotiorum* CGF on various media.

Media	Colony surface	Reverse color	Colony diameter (mm)	
			25°C	37°C
CYA	Circular, planar, thin to felty, sulcate at the center, produced hyaline exudation, pale yellow	Pale yellow	49	13
CY20S	Circular, planar, thin to felty, immersed, creamy yellow	Pale yellow	62	42
CZ	Circular to irregular, plane, thin, raised at the center, pale yellow	Creamy yellow	25	9
MEA	Circular, planar, thin, yellow-brown	Dark yellow	42	10
PDA	Circular, planar, thin, yellow-brown	Yellow-brown	41	9
CMA	Circular, planar, appressed, white	Hyaline	23	4
OMA	Circular, planar, thin to felty, produced hyaline exudation, yellow-brown	-	33	6
SDA	Circular, thin to felty, raised at the center, radially sulcate, produced hyaline exudation, yellow	Yellow	52	8
YMEA	Circular, planar, thin to somewhat cottony, produced hyaline exudation, creamy yellow	Yellow-brown	39	10
MY20	Circular, planar, thin to felty, yellow-brown	Pale yellow	56	36

Macroscopic characteristics were observed on various media cultured at 25°C for 7 days. Colony diameter was measured at 25°C and 37°C after 7 days.

characteristics on the four media: CYA, CY20S, CZ, and MEA. All strains achieved similar growth on the same media at both 25°C and 37°C for 7 days. Strains grew rapidly at 25°C and restrictedly at 37°C with a colony diameter of 0.8-1.5 cm, except for CY20S (2.2-4.3 cm). The size of the sclerotium of NRRL 4901 was bigger than those of NRRL 415 (type strain), while the sclerotium size of CGF was smaller than those of the other two strains. *A. sclerotiorum* NRRL 415 sporulated better than the other strains. The sporulation of the three strains on CZ and

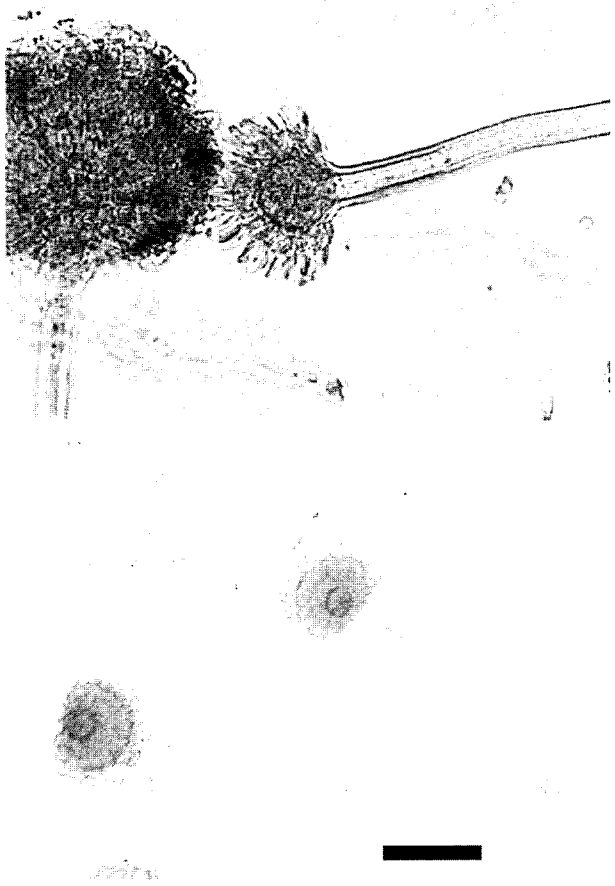

Fig. 2. Microphotograph of *A. sclerotiorum* CGF, having conidial heads and conidiophore (Top: ×1,000, Bottom: ×400, Bar: 100 μm).

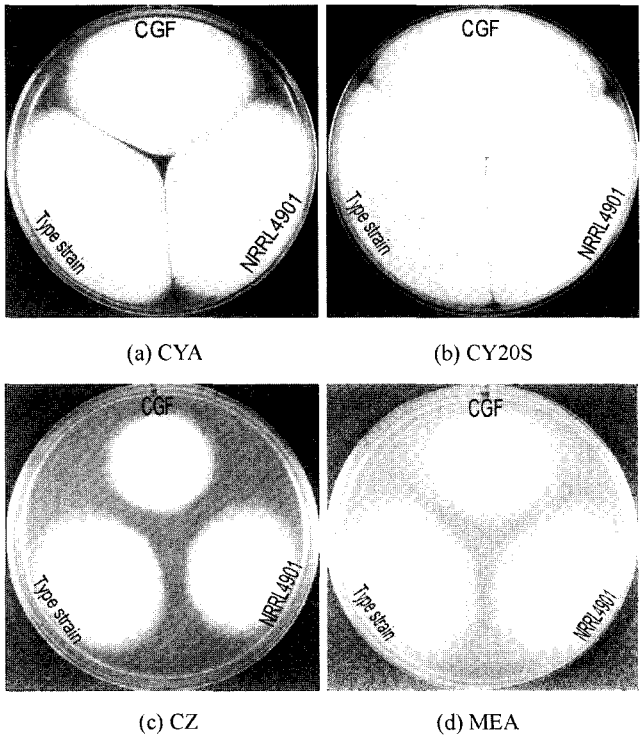

Fig. 3. Comparative growth morphology of three *A. sclerotiorum* strains after 1 week at 25°C.

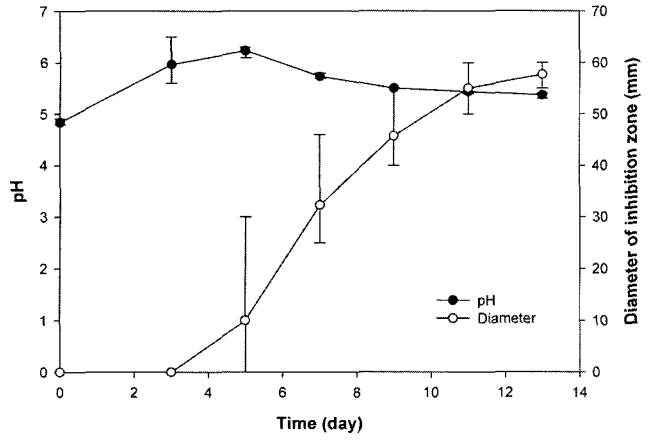

Fig. 4. Time-course profiles of the production of antifungal agent by *A. sclerotiorum* CGF using potato dextrose broth (PDB). Loading volume: 50 µl of culture filtrate/paper disc. Incubation temperature and time: 25°C, 5 days. Test organism: *P. drechsleri.*

MEA was very low. In particular, *A. sclerotiorum* NRRL 4901 showed the lowest sporulation on all media (Fig. 3). Based on the above results, this strain was identified as *Aspergillus sclerotiorum* and renamed as *Aspergillus sclerotiorum* CGF.

A. sclerotiorum CGF was cultured on PDB, and the amount of antifungal agent produced was then evaluated by the diameter of inhibition zone by the paper disc diffusion method against *P. drechsleri.* The time-course profile of the production of antifungal agent is shown in Fig. 4. The pH was increased from the initial 4.9 to 6.2 when antifungal agent was produced, and then decreased finally to 5.4. Antifungal agent was produced after 5 days of incubation, and the highest antifungal activity was obtained after 11 days of fermentation.

To evaluate the antifungal activity of *A. sclerotiorum* CGF against various phytopathogenic fungi, 50 µl of culture filtrate were loaded on the paper discs and placed at 3 cm away from agar pieces of phytopathogenic fungi. Among the phytopathogenic fungi tested, the inhibition of growth

Table 3. Antifungal activity of culture filtrate of *A. sclerotiorum* CGF against phytopathogenic fungi.

Strains	Antifungal activity (mm)
Aspergillus flavus KACC 40250	-
Botrytis cinerea KACC 40573	-
Fusarium oxysporum KACC 40053	-
Fusarium solani KACC 40384	-
Phytophthora infestans KACC 40718	27
Pythium ultimum KACC 40705	-
Rhizoctonia solani AG-1 KACC 40101	-
Sclerotium rolfsii KACC 40832	-
Trichoderma harzianum KACC 40784	-

Loading volume: 50 µl of culture filtrate/paper disc. Incubation temperature and time: 25°C, 5-7 days.

Table 4. Antifungal activity of culture filtrate of *A. sclerotiorum* CGF.

Strain	Diameter of inhibition zone (mm)
Phytophthora drechsleri	52
Phytophthora capsici	42
Phytophthora cambivora	50
Phytophthora cactorum	41
Phytophthora infestans	68
Phytophthora nicotianae	62

Loading volume: 50 µl of culture filtrate/paper disc. Incubation temperature and time: 25°C, 5 days.

was observed only with *Phytophthora infestans.* The inhibition distance of growth was 27 mm against *P. infestans* (Table 3). The diameter of inhibition zone of six *Phytophthora* species was measured using the paper disc diffusion method. As shown in Table 4, the growth of *Phytophthora* species was greatly inhibited by the antifungal agent. In addition, the inhibition of germination of *P. capsici* zoospore is shown in Fig. 5.

The antifungal agent was very stable in a wide range of pH levels. Up to 96% of the antifungal activity was maintained at pH 10 and 11, and the antifungal agent was also very stable during the reaction time at 80°C, maintaining 90% of the antifungal activity for 6 h (Fig. 6). The antifungal agent produced by *Aspergillus sclerotiorum* CGF was very effective and unique in inhibiting the mycelial growth and zoospore germination of *Phytophthora* species. These results, therefore, clearly indicate that the antifungal

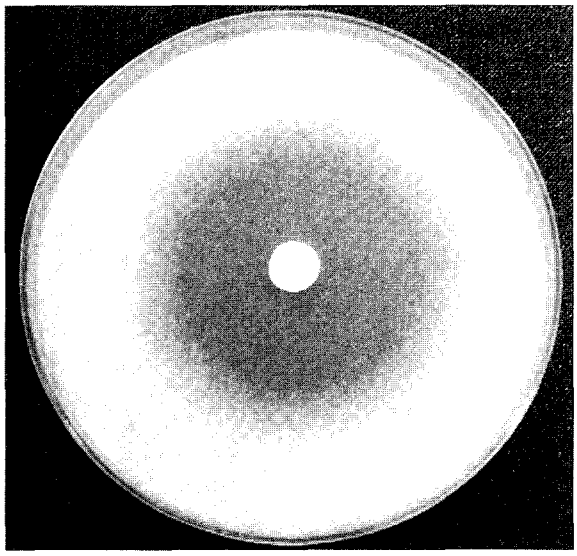

Fig. 5. The germination of *P. capsici* zoospores was inhibited by the culture filtrate of *A. sclerotiorum* CGF. Loading volume: 50 µl of culture filtrate/paper disc. Incubation temperature and time: 25°C, 5 days. Inoculation concentration of zoospore: 2×10^5 spore^{-1}.

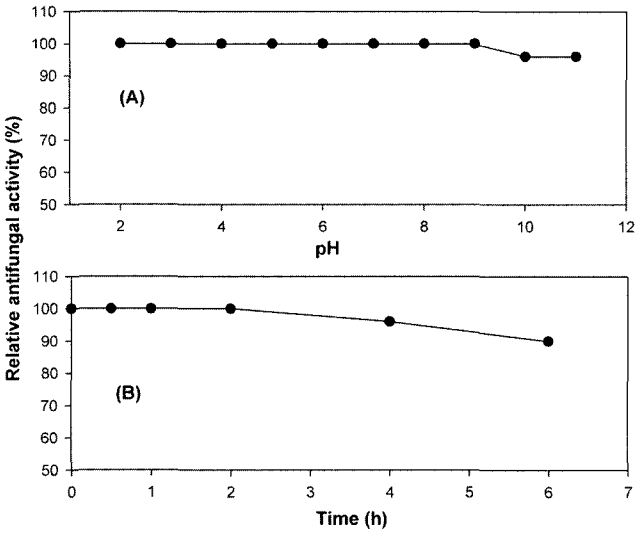

Fig. 6. pH (A) and thermal (B) stabilities of the antifungal agent of *A. sclerotiorum* CGF.
Loading volume: 50 µl of culture filtrate/paper disc. Incubation temperature and time: 25°C, 5 days. Test organism: *P. drechsleri*.

agent produced by *A. sclerotiorim* CGF has potential to biocontrol *Phytophthora* diseases.

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