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Article

Azalomycin F Complex Is an Antifungal Substance Produced by *Streptomyces malaysiensis* MJM1968 Isolated from Agricultural Soil

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MJM1968, an actinomycete showing potent antifungal activity, was isolated and identified by 16S rDNA sequence analysis as *Streptomyces malaysiensis.* MJM1968 showed strong antifungal activity on phytopathogenic fungi such as *Fusarium oxysporum*, *Rhizoctonia solani*, *Cladosporium cladosporioides*, *Fusarium chlamydosporum*, *Colletotrichum gloeosporioides* KACC 40693, *Alternaria mali* KACC 40026, and *Pestalotia* spp. KACC 40501, *in vitro*. An antifungal compound was isolated from culture filtrate of strain MJM1968 by a series of chromatographic methods. Treatment of agricultural soil with strain MJM1968 mycelia reduced the native fungal population by more than 60% after 14 days. Treatment of soil with 10 mg/mL partially purified extract lowered native fungal density by more than 80% after 14 days. The compound, which exhibited antifungal activity in a broad range of pH and temperature, was identified as azalomycin F complex by ^{1}H and ^{13}C NMRs. This is the first report on the isolation of azalomycin F complex from *S. malaysiensis,* demonstrating a broad-spectrum suppression of fungal pathogens in agricultural soil.

Key words: antifungal activity, azalomycin F complex, biological control, MJM1968, pathogen suppression

Soilborne fungal pathogens pose a great threat to crop production in tropical and temperate regions worldwide. They are also recalcitrant to most control measures compared with pathogens that attack the above-ground portions of plants [Raaijmakers *et al*., 2009]. Many diseases that are caused by soilborne fungal pathogens are difficult to diagnose, because the symptoms in above-ground plant parts are indistinct or similar to those caused by abiotic stresses [Raaijmakers *et al*., 2009]. Infections from multiple soilborne fungal pathogens can result in diseases complex [Martin, 2003]. In addition, the soil environment is complex, making it difficult to understand all aspects of diseases that are caused by soilborne fungal pathogens. Soil fumigation with methyl bromide has long been used as a viable strategy to control a broad range of pathogens [Martin and Bull, 2002], but its use has waned following reports on its ozone-depleting effects and toxicity in nontarget organisms. Systemic fungicides, such as phenylamides and phosphonates, are new chemical control agents of soilborne fungal pathogens; however, they create many problems, including biodegradation in soil and the emergence of resistant pathogens [Lucas *et al*., 1990].

Streptomyces, soil-dwelling filamentous bacteria, are prolific producers of a wide range of antimicrobial agents [Watve *et al*., 2001]. To this end, Streptomyces was evaluated against plant diseases to develop biocontrol agents, based on the strain or its antibiotic as an active ingredient. Cycloheximide and streptomycin, produced in *S. griseus,* were the first antibiotics used to control fungal and bacterial diseases in plants, respectively [Leben and Keitt, 1954]. Since then, many reports have shown the use of antibiotics from Streptomyces strains in controlling plant diseases. Antifungal antibiotics from Streptomyces that have been developed into fungicides for agricultural use include, blasticidin S [Takeuchi *et al*., 1957], Kasugamycin [Umezawa *et al*., 1965], validamycin [Iwasa *et al*., 1970], polyoxin [Isono *et al*., 1965],

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bleomycin [Shimura *et al*., 1970], gopalamicin [Nair *et al*., 1994], tubercidin [Hwang and Kim, 1995], phenylacetic acid [Hwang *et al*., 2001], and jinggangmycin [Shen, 1996]. Streptomyces has also been used as live mycelium or spores in biofungicide preparations. The commercial preparation Mycostop™ contains spores of *S. griseoviridis* strain K61 as the active ingredient for use against soilborne fungal pathogens, such as *Pythium* spp., *Phytophthora* spp., *Fusarium* spp., and *Rhizoctonia* spp. [Minuto *et al*., 2006].

In the present study, *Streptomyces malaysiensis* strain MJM1968 was isolated from Imsil, Korea and was found to have potent antifungal activity by screens against phytopathogenic fungi *in vitro*. We evaluated the efficacy of strain MJM1968 in suppressing phytopathogenic fungi in agricultural soil of Korea.

Materials and Methods

Soil collection, microbial isolation and culture. Strain MJM1968 was isolated from Imsil, Korea and was deposited into and maintained at the Extract Collection of Useful Microorganisms (ECUM) library at Myongji University, a member of the Korean National Research Resource Center (KNRRC).

Agricultural soil was collected from a yam field in Yeoju, Korea -a region heavily infected with fungus- sifted through a 2-mm mesh sieve to remove large solid particles, and stored at 4°C until use. This soil was used to isolate fungal pathogens and for *in vivo* study of the suppression of pathogenic soil-inhabiting fungi by strain MJM1968 and the active compound. The soil samples were suspended in 10 mL saline (0.85% NaCl). Subsequently, 0.1 mL of the suspension was plated onto potato dextrose agar (PDA) plates that were supplemented with the antibacterial agents bacitracin (60 μg/mL), kanamycin (100 μg/mL), and nalidixic acid (50 μg/mL) and incubated at 28°C for 3 days. Fungal colonies were selected based on morphology and subcultured on PDA for further study. *Colletotrichum gloeosporioides* KACC40693, *Pestalotia* spp.KACC40501, *Penicillium digitatum* KACC 40822, *Alternaria mali* KACC 40026, *Rhizobium meliloti* KACC 10025, *Streptomyces scabiei* KACC 20101, and *Pseudomonas syringae* pv. *syringae* KACC 10134 were obtained from the Korean Agricultural Culture Collection (KACC), National Institute of Agricultural Biotechnology, Korea.

Genomic DNA preparation, amplification, and sequencing of 16S rDNA and phylogenetic analysis of MJM1968. Genomic DNA was prepared by using Genomic DNA preparation kit (Corebio, Seoul, Korea) according to the manufacturer's protocol, and 16s rDNA was amplified by using the following primers: 27F (forward), 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R (reverse) 5'-TACGGYTACCTTGTTACGACTT-3' [Coombs and Franco, 2003]. The PCR product was purified and directly sequenced. Related sequences were obtained from the GeneBank database (National Center for Biotechnology Information, National library of medicine) using the BLAST search program. The sequence was aligned with those of the reference strains using Clustal X version 1.8 [Thompson *et al*., 1997]. A phylogenetic tree was constructed by the Neighbor-joining method [Saitou and Nei, 1987] using MEGA 4.0 software [Tamura *et al*., 2007]. Gaps were treated by pairwise deletion and bootstrap analysis was done by using 1,000 pseudoreplications.

Fungal DNA isolation, amplification, and sequencing of 18S rDNA. Fungal DNA was isolated using fungal genomic DNA preparation kit, SolGent, Seoul, Korea, following the manufacturer's protocol. The genomic DNA from each fungal isolate was subjected to PCR for the amplification of 18S rRNA gene using ITS1 and ITS4 [White *et al*., 1990]. The PCR products were subjected to 1% agarose gel electrophoresis and purified using CoreBio gel extraction kit (CoreBio). The sequences of the amplified products were determined with the forward (ITS1) and reverse (ITS4) primers using ABI 3730XL capillary DNA Sequencer (50-cm capillary).

***In vitro* assay for antagonist activity.** Strain MJM1968 was evaluated for antagonist activity against *F. oxysporum*, *R. solani*, *C. cladosporioides*, *F. chlamydosporum*, *C. gloeosporioides* KACC 40693, *A. mali* KACC 40026, and *Pestalotia* spp. KACC 40501 by dual-culture *in vitro* assay on PDA plates. MJM1968 was inoculated onto PDA by streaking 1 cm from the edge of each Petri dish. After incubation at 28°C for 3 days, an agar block (0.5 cm in diameter) that contained mycelial mats of the respective fungus, obtained from 3-day-old cultures, was used to inoculate each dish 5 cm from MJM1968. Fungal plugs were also placed on uninoculated PDA plates as control. The dishes were incubated at 28°C for 7 days and examined for a zone of inhibition between the fungus and MJM1968. The level of inhibition was determined as reported previously [Yuan and Crawford, 1995].

Fermentation, fractionation, and partial purification of the active compound. Strain MJM1968 was cultured in BN liquid medium in 2-L baffled flasks for 7 days at 200 rpm. The culture filtrate was collected by centrifugation, and its antifungal activity was confirmed against *R. solani*. Approximately 40 L of culture filtrate was prepared and partially purified by passage through a glass column (15 L, 140×550 mm) packed with 10 L Diaion HP-20 (Mitsubishi Chemical, Tokyo, Japan). The column was

eluted sequentially with H_2O, 50% methanol, 100% methanol, 50% acetone, and 100% acetone. The fraction with antifungal activity was concentrated, applied to an RP-18 open column, and eluted sequentially with 50% methanol, 60% methanol, 70% methanol, 80% methanol, 90% methanol, 100% methanol, 100% acetone, 100% ethyl acetate, and 100% hexane. After vacuum-drying, 5 mg of each fraction was tested against *F. oxysporum* and *R. solani* by disc diffusion assay. The fraction that contained antifungal activity was used for further study.

Effect of MJM1968 on fungal density in agricultural soil. MJM1968 cultured in BN liquid medium for 3 days and the active fraction that had antifungal activity were used for soil treatment. The mycelia were washed with 0.85% NaCl and diluted to treat the soil. There were 3 treatment categories and a control, each containing 10 g of soil. Soil from each treatment category was supplemented with 1 mL suspension of MJM1968 that contained 6.6×10^2, 6.6×10^3 or 6.6×10^4 cfu/mL; or 1 mL of the partially purified compound that contained 100, 1000 or 10,000 μg/mL; the control received 0.85% NaCl or sterile distilled water. The treated soil was incubated at 28°C and plated on PDA to determine the number of fungal colony-forming units. Fungal density was determined at 0 (before treatment), 7, and 14 days after treatment.

Isolation and identification of active compound from MJM1968. Fractions that had antifungal activity were purified on a semi preparative HPLC system that was equipped with a C18 reverse phase column (YMC-Pack ODS-A, 5 μm, 250×10 mm, Waters), using an elution system under isocratic conditions (77% methanol in distilled water) at a flow rate of 2 mL/min, and detected on RI and UV detectors at dual wavelengths of 254 and 365 nm. Active fractions were collected and subjected to a second round of preparative HPLC for further purification under the following conditions: C18 reverse phase column (YMC-Pack ODS-A, 5 μm, 250× 10 mm), acetonitrile: H_2O; 65:35, flow rate 3 mL/min. The purified compound was dissolved in methanol. ^{1}H-NMR spectra were recorded on a Bruker Avance DPX 600 MHz NMR spectrometer (Bruker, Karlsruhe, Germany), and ^{13}C-NMR spectra were recorded on a 300 MHz NMR spectrometer (Bruker). Chemical shifts are given in ppm, using tetramethylsilane (TMS) as an internal standard. FAB-MS spectra were measured on a JMS-AX 505 WA spectrometer (JEOL, Tokyo, Japan).

pH and thermal stability of the purified compound from strain MJM1968 culture filtrate. For the pH stability test, the pH of the purified compound was adjusted to pH 1-14 and set aside for 3 h before being readjusted to neutral. To measure thermal stability, the compound was exposed to various temperatures -40, 60, and 80°C for 60 min and 100 and 120°C for 30 min- and cooled to room temperature. Two hundred microliters of each treatment was spotted onto paper discs, and the activity against *R. solani* was determined by agar diffusion assay. All the treatments were administered in three independent replicates.

Inhibitory spectrum of the purified compound. The purified compound was tested for its inhibitory activity on bacterial and fungal pathogens. Bacterial phytopathogens were tested by top cover assay. The bottom layer was 1% nutrient agar, and the top layer was 0.7% nutrient agar supplemented with 1% bacterial inoculum, which was precultured in nutrient broth until OD_{600}=1.0. One hundred microliters of the fungal spore suspension (1×10^6 spores/ mL of the test fungus) was spread onto PDA plates. Paper discs containing 50 μg of the purified compound were placed on fungi- and bacteria-inoculated plates. The zone of inhibition was measured after 24 to 72 h of incubation at 28°C, depending on the strains.

Results

Isolation and identification of fungi isolated from soil. A total of 156 fungi were isolated from the soil collected from the yam cultivation field, Yeoju. Twelve fungi observed to occur high in numbers were subjected to 18S rRNA gene sequence analysis. The fungi were identified based on the 18S rRNA sequence by comparison with that of known fungi using the NCBI-MBLAST program (Table 1). Among the 12 strains, 4 strains were identified as *Fusarium* species, and 7 strains were identified as known plant pathogens (Table 1).

Phylogenetic analysis of MJM1968 based on 16s rRNA gene sequence. The 16S rRNA gene was amplified by PCR. The PCR product was sequenced, and the sequences were aligned using the Clustal X ver 1.8 multiple alignment program. The 16S rRNA gene sequence analysis showed that the strain MJM1968 shared 98% homology with *Streptomyces malaysiensis* Da08002 (EU595361) (Fig. 1).

Antifungal activity of MJM1968 against phyto-pathogenic fungi. The *in vitro* antifungal activity of MJM1968 on phytopathogenic fungi was determined by measuring the width of the zone of fungal growth inhibition. MJM1968 showed robust inhibitory activity against all fungi examined. The inhibition zone was maximum for *F. oxysporum* and *R. solani* (15 mm), followed by *A. mali* KACC 40026 (13 mm), *C. gloeosporioides* KACC 40693 (12 mm) and *C. cladosporioides* (10 mm). MJM1968 showed weaker antifungal activity on *Pestalotia* sp. KACC 40501 (8 mm) and *F. chlamydosporum* (6 mm).

Table 1. Identification of representative fungi isolated from agricultural soil based on 18S rRNA gene sequence

Isolate	Homology (%)	Strain name	Phytopathogen	Reference
YS-01	99	*Penicillium pinophilum*	-	
YS-23	99	*Cladosporium cladosporioides*	YES	Wicklow and Poling, 2009
YS-32	100	*Fusarium equiseti*	YES	Fajola, 1979
YS-34	99	*Trichocladium asperum*	-	
YS-52	99	*Fusarium oxysporum*	YES	Fajola, 1979
YS-61	99	*Alternaria ochroleuca*	-	
YS-70	99	*Eupenicillum brefeldianum*	-	
YS-78	99	*Rhizoctonia solani*	YES	Fajola, 1979
YS-99	99	*Penicillium raperi*	-	
YS-110	99	*Fusarium chlamydosporum*	YES	Morales-Rodríguez *et al*., 2007
YS-112	99	*Fusarium cerealis*	YES	Stepieñ, 2008
YS-134	100	*Cladosporium tenuissimum*	YES	Fajola, 1979

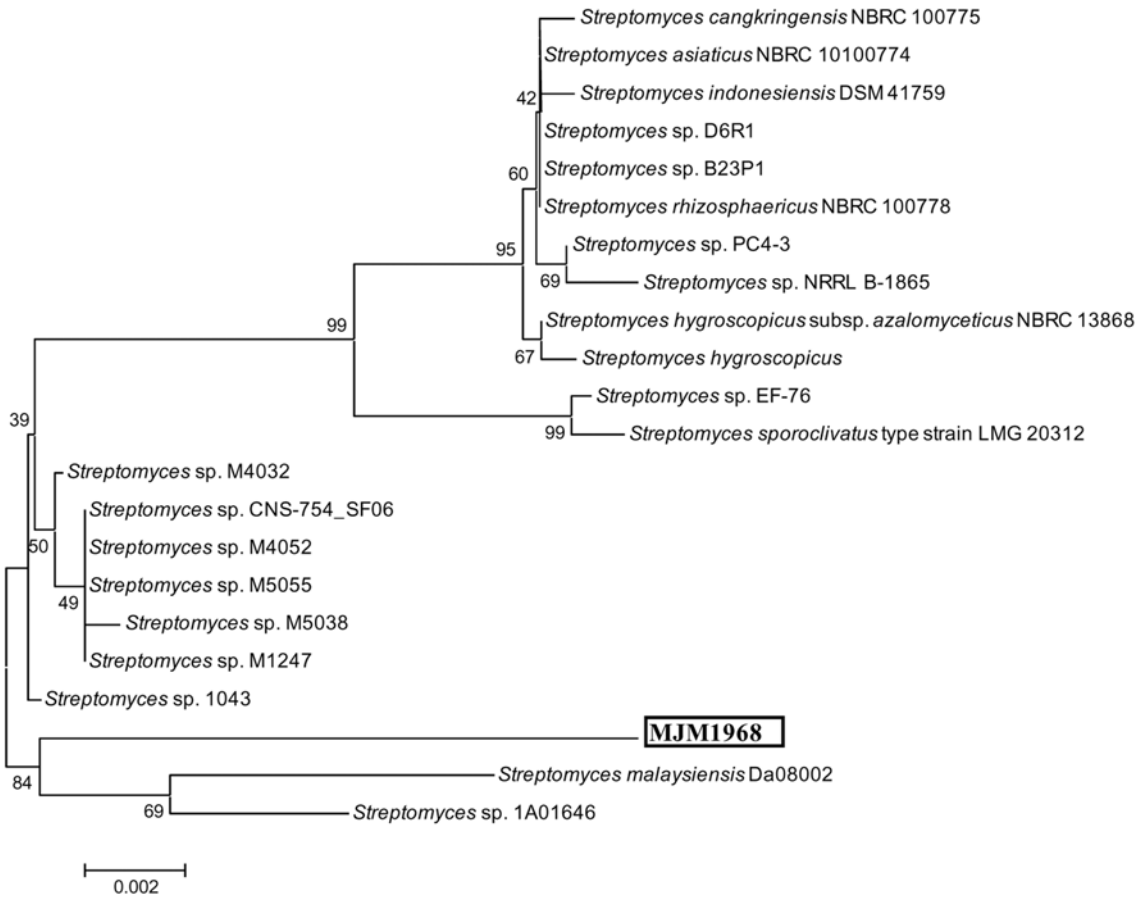

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence showing the relationship between strain MJM1968 with other *Streptomyces* spp. The tree was constructed using the neighbor-joining method using MEGA4.0 program. The bootstrap values were evaluated from 1,000 replications.

Effect of strain MJM1968 on fungal density in agricultural soil. Treatment of soil with MJM1968 mycelia inhibited the fungal inhabitants and reduced fungal density. Seven days after treatment, fungal density decreased to 77% at 6.6×10^2, 42% at 6.6×10^4, and 40% at 6.6×10^6 cfu/mL. Fourteen days after treatment, fungal density decreased by more than 60% under all treatment conditions (Fig. 2A).

Soil treated with 100 μg/mL MJM1968 of partially purified extract showed a decrease in fungal density to 90% compared with the untreated control after 7 days and to 80% after 14 days. When the soil was treated with

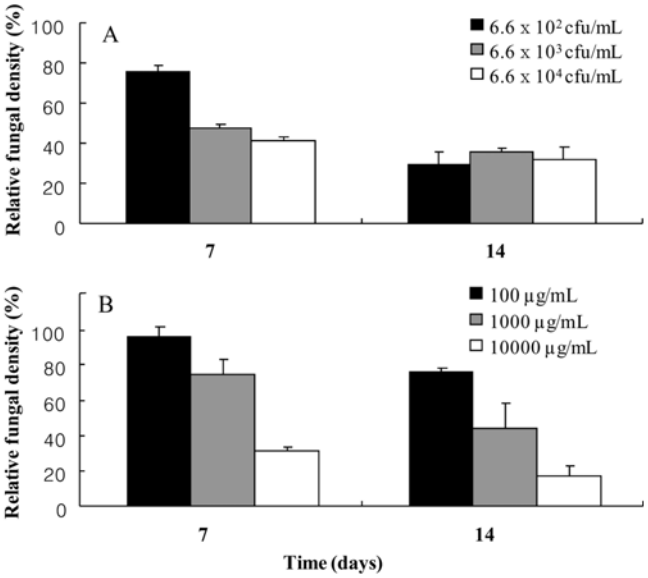

Fig. 2. Effects of MJM1968 mycelia (A) and the partially purified extract (B) on soil fungal density. Soil was treated with MJM1968 and the partially purified extract at the concentrations indicated. Total fungal density was measured 7 and 14 days after treatment.

1000 μg/mL purified extract, the fungal density decreased to 75% after 7 days and to 43% after 14 days. A significant decrease in total fungal density in the soil was observed at 10,000 μg/mL of extract treatment, and fungal density decreased to 30% compared with the untreated control after 7 days and to 18% after 14 days (Fig. 2B).

Isolation and identification of active compound from MJM1968. Of the fractions that were eluted from the Diaion HP-20 column, the 100% methanol-eluted fraction was found to have antifungal activity. This fraction was vacuum-concentrated and subjected to silica gel RP-18 chromatography. Of the nine fractions from reverse phase flash chromatography, the acetone-eluted fraction was chosen for further purification due to its antifungal activity. From the first preparative HPLC, the active compound was eluted at a retention time of 45 min. This fraction was collected and applied to a second round of preparative HPLC. Approximately 87 mg of active compound (1968-rfc7-rp19-rp2) was eluted at a retention time of 6-7 min (data not shown).This active compound was determined to be azalomycin F complex by NMR spectroscopy (Fig. 3). The compound generated a molecular ion peak at *m/z* 1064, 1082, and 1096, closer to the components of azalomycin F complex, F3a (MW1067), F4a (MW 1081), and F5a (MW 1095), respectively [Chandra and Nair, 1995]. The ^{1}H-NMR and ^{13}C-NMR (Table 2) signals of the active compound (1968-rfc7-rp19-rp2) were compared to that of azalomycin

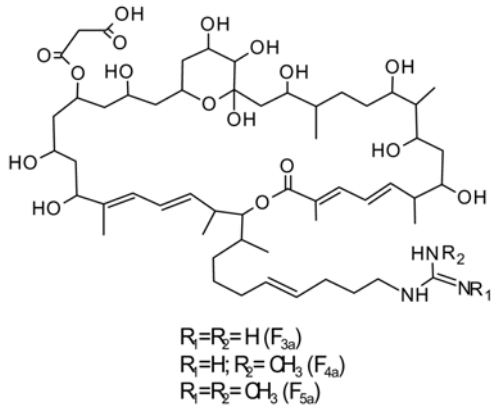

Fig. 3. Structure of azalomycin F complex, the active antifungal compound from MJM1968.

F complex [Chandra and Nair, 1995] and were found to be identical.

pH and thermal stability of the purified compound. High antifungal activity was observed at neutral pH; acidic treatment and alkaline pH had minor effects on antifungal activity of the compound (Fig. 4A). At high acidity (pH 1), 80% of the antifungal activity remained. The antifungal activity was stable at acidic pH up to 2 and declined at pH 1. A gradual reduction in antifungal activity was observed at alkaline pH starting at pH 10. Nevertheless, under strong alkaline conditions (pH 14), 70% of the antifungal activity was retained. These findings demonstrate the high stability of antifungal activity over a wide range of pH (Fig. 4A).

Antifungal activity was stable at 40, 60, and 80°C for 1 h. A slight reduction in activity was observed after incubation at 100°C for 30 min, at which 89.5% of the activity was retained. Exposure of the compound to 121°C for 30 min reduced the antifungal activity to 68.4% (Fig. 4B). These results suggest that the antifungal activity is stable at high temperatures.

Inhibitory spectrum of the purified compound. The purified compound from MJM1968 had robust activity against phytopathogenic fungus and Gram-positive bacteria, but not Gram-negative bacterial strains. The compound showed high efficacy against *C. gloeosporioides* KACC 40693, *R. solani*, *A. mali* KACC 40026, and *Pestalotia* spp. KACC 40501. The compound showed moderate activity against *S. scabiei* KACC 20101, *F. oxysporum*, *P. pinophilum*, and *P. digitatum* KACC 40822 (Table 3).

Discussion

Most research on biocontrol agents has focused on plant disease suppression rather than pathogen suppression. Pathogen suppression develops in bulk plant-free soil or

Table 2. Comparison of ^{13}C-NMR data of the active compound isolated from strain MJM1968 with those of azalomycin F complex

Carbon	Azalomycin F* (δ)	1968-rfc7-rp19-rp2 (δ)	Carbon	Azalomycin F* (δ)	1968-rfc7-rp 19-rp2 (δ)
C-1	170.02	168.623	C-31	125.09	123.709
C-2	126.76	125.301	C-32	128.47	127.145
C-3	140.07	138.685	C-33	136.11	134.752
C-4	127.50	126.193	C-34	40.84	39.534
C-5	145.98	144.634	C-35	80.85	79.242
C-6	40.64	39.378	C-36	33.52	32.202
C-7	75.78	74.327	C-37	28.35	26.955
C-8	39.27	37.801	C-38	27.85	26.476
C-9	75.19	73.494	C-39	30.52	29.175
C-10	44.10	42.672	C-40	132.49	131.064
C-11	72.33	70.899	C-41	130.15	128.796
C-12	39.27	37.801	C-42	29.77	28.365
C-13	29.77	28.365	C-43	30.52	29.175
C-14	40.64	39.378	C-44	42.14	40.657
C-15	72.49	71.056	C-45	12.84	11.441
C-16	41.99	40.523	C-46	16.89	15.672
C-17	99.79	98.315	C-47	10.54	9.009
C-18	77.39	75.702	C-48	14.94	13.344
C-19	69.69	68.259	C-49	13.33	11.835
C-20	41.16	39.779	C-50	17.68	16.140
C-21	65.58	64.035	C-51	14.38	12.898
C-22	41.89	40.389	C-52 (3a)	158.69	157.200
C-23	65.71	64.697	C-52 (4a)	158.27	156.799
C-24	44.55	43.066	C-52 (5a)	157.30	155.855
C-26	46.26	45.051	C-53	28.34	26.952
C-27	66.32	64.753	C-54	171.59	170.140
C-28	44.02	42.583	C-55	46.26	45.051
C-29	74.28	72.743	C-56	173.98	172.646
C-30	140.07	138.685	C-57 (5a)	28.40	26.960

* ^{13}C-NMR chemical shift reported by Chandra and Nair, 1995

independently from any influence of the plant, whereas disease suppression occurs in the presence of the plant [Termorshuizen and Jeger, 2008]. Suppression of pathogens in the agricultural field can lead to low disease incidence, and disease suppression can be achieved indirectly. In the present study, the antifungal activity of an actinomycete strain, MJM1968, was identified and studied for its effect on the fungal pathogens in agricultural soil. 16S rRNA gene sequence showed 98% sequence identity with that of *S. malaysiensis* Da08002 (EU595361). We evaluated the effects of strain MJM1968 and the partially purified extract on pathogen suppression in agricultural soil from a yam cultivation area in Yeoju, Korea, where the populations of phytopathogenic fungi that cause rot disease in Yam tubers were found to be high. MJM1968 was observed to be an antagonist of phytopathogenic fungi in soil from Yeoju. Administration of the partially purified extract decreased the native fungal density in a dose-dependent manner, but required repeated application. Nevertheless, treatment with the cultured strain reduced fungal density dose-dependently after 1 week, but after 2 weeks, the extent of reduction was more pronounced and nearly equal at all concentrations, suggesting that the suppressive activity of the strain was not limited to antibiosis and that other mechanisms, such as competition and hydrolytic enzymatic activity, are involved.

Similar findings have been reported [Bowe and Locke, 2000; 2004], wherein formulated plant extracts was fed to soil, resulting in lowering the population density of *F. oxysporum*, *P. nicotianae,* and *V. dahlia*; however, the active compound was not identified. Chung *et al*. [2005]

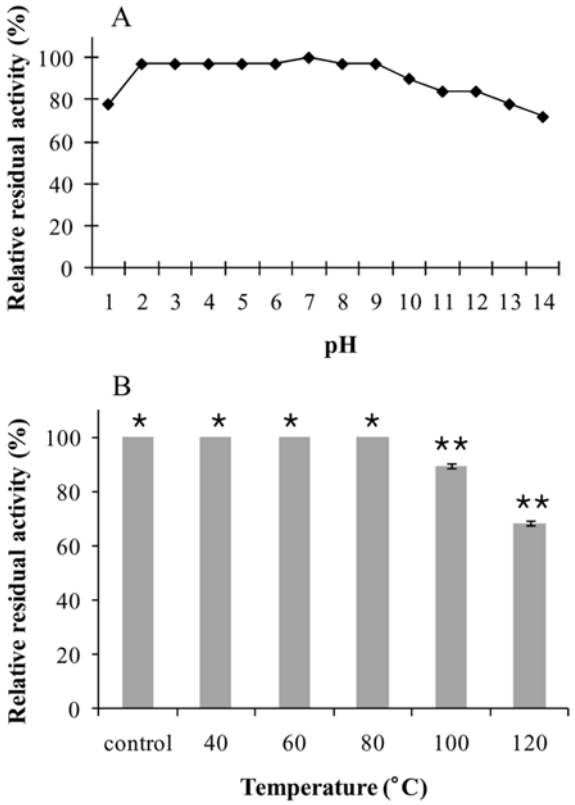

Fig. 4. pH and thermal stability of azalomycin F complex, the purified compound, from MJM1968. (A) A solution of the purified compound was adjusted to various pH conditions, incubated for 60 min, and readjusted to neutral for test. Values presented are averages of three independent replicates. (B) The purified compound was exposed to various temperatures for 60 (*) or 30 min (**). Residual activity was expressed as the percentage of activity shown by untreated compound.

reported the formulation of a soil bio-fungicide, based on *S. padanus* and *S. xantholiticus,* against damping-off, caused by *R. solani*. Nevertheless, only few reports are available on the use of antibiotics from Streptomyces for soil applications.

The antifungal activity was further purified and identified as azalomycin F complex. The purified compound had robust antifungal activity on all tested fungal pathogens. The compound also exhibited stability and antifungal activity on a broad range of pH and temperature, which is a prerequisite for testing the compound under field conditions.

We conclude that azalomycin F complex is the principal component responsible for the antifungal activity of strain MJM1968, by suppressing the population density of fungal pathogens in the Korean agricultural soil. Further studies on its formulation and large-scale field studies are underway to develop a stable soil bio-fungicide based on the strain MJM1968 and azalomycin F complex.

Table 3. Antimicrobial activity of azalomycin F complex, the purified compound, on phytopathogens‡

Microorganisms	Activity (mm)*
Penicillium pinophiloum	17
Rhizoctonia solani	32
Fusarium oxysporum	21
Colletotrichum gloeosporioides KACC40693	40
Pestalotia spp.KACC40501	30
Penicillium digitatum KACC 40822	17
Alternaria mali KACC 40026	33
Streptomyces scabiei KACC20101	25
Rhizobium meliloti KACC 10025	-
Pseudomonas syringae pv. *Syringae* KACC10134	-

‡50 μg of purified compound was administered to each test strain

*Activity is expressed as diameter of the zone of inhibition -No inhibition

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