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Antifungal Activity of β-Asarone from Rhizomes of *Acorus gramineus*

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An antifungal substance was isolated from the extract of *Acorus gramineus* using various chromatographic procedures. The antibiotic was identified as β-asarone, *cis*-2,4,5-trimethoxy-1-propenylbenzene, on the basis of the high-resolution EI-mass, NMR, and UV spectral data. β-Asarone completely inhibited mycelial growth of some plant pathogenic fungi, *Cladosporium cucumerinum*, *Colletotrichum orbiculare*, *Magnaporthe grisea*, and *Pythium ultimum*, in a range of 0.5−30 μg/mL. The growth of *Bacillus subtilis*, *Erwinia carotovora* subsp*. carotovora*, *Ralstonia solanacearum*, and *Xanthomonas campestris* pv. *vesicatoria* was slightly suppressed by β-asarone. As the concentration of β-asarone increased, *M. grisea* infection was drastically inhibited on rice leaves. Treatment with 500 μg/mL of β-asarone also greatly suppressed lesion formation of *Co. orbiculare* on cucumber leaves. This is the first study to demonstrate in vitro and in vivo antifungal activity of β-asarone against plant fungal pathogens *M. grisea* and *C. orbiculare*.

KEYWORDS: ***Acorus gramineus*; β-asarone; antifungal activity; cucumber anthracnose; rice blast**

INTRODUCTION

The search for new natural products to control human and crop diseases and pests is a promising area of research. Natural products are likely to be a source of new commercially used biofungicide leads (*1*). The chemical novelty associated with natural products is higher than that of any other source. Natural compounds produced by the secondary metabolism of plants are available for the development of practically useful fungicides effective against plant pathogens.

Acorus gramineus Solander of the family Araceae has been incorporated into traditional medicine in Asia. In particular, the dry rhizome of *A. gramineus*, called Acori graminei rhizoma (AGR), is used extensively in Korean traditional medicine. Both Korean and Chinese pharmacopoeias have demonstrated that *A. gramineus* exhibits sedative, digestive, analgesic, diuretic, and antifungal actions (*2*−*4*). The volatile oil (0.11−0.42%) of *A. gramineus* mainly consists of β-asarone (63−81%), α-asarone (8−14%), caryophyllene (1−4%), isoasarone (0.8−3.4%), methylisoeugenol (0.3−6.8%), safrole (0.1−1.2%), and asaryl aldehyde as major components (*3*−*5*).

Much work has been done to investigate the biological activity of *A. gramineus*. The water-extracted decoction, ethanol extract, and volatile oil of *A. gramineus* were found to induce diverse actions such as sedation, prolongation in pentobarbital-induced sleeping time, and attenuation of apomorphine-induced stereotypic behavior in mice (*6*, *7*). Anthelmintic and pesticidal activities of *A. gramineus* have been reported to be associated with the phenylpropanoids α- and β-asarones (*8*). α- and β-Asarones, the major essential oil components in *A. gramineus*, exhibited neuroprotective action against the excitotoxicity induced by *N*-methyl-D-aspartate (NMDA) or glutamate (Glu) in cultured rat cortical cells (*9*). β-Asarone isolated from the rhizome of a related species, *Acorus calamus*, was shown to have anthelmintic and antibacterial activities against *Bacillus subtilis* and *Staphylococcus aureus* (*10*). The compound β-asarone is known to be toxic (*11*, *12*).

In the search program for the antifungal compounds from medicinal plants, we have isolated and identified antifungal compound β-asarone from *A. gramineus* This paper reports the isolation procedures of β-asarone, its molecular characteristics, and the in vitro and in vivo antifungal activity against plant pathogenic fungi.

MATERIALS AND METHODS

Experimental Procedures. Column chromatography was performed in silica gel ($60F_{254}$, 63−200 μm, Merck) and Sephadex LH-20 (26 × 950 mm C26/100 column or 10 × 950 mm C10/100 column packed with Sephadex LH-20 resin, Pharmacia, Uppsala, Sweden). Preparative thin-layer chromatography (TLC) was performed on glass plates (20 × 20 × 0.2 mm) coated with silica gel (60 GF_{254}, Merck). The spots of antifungal compounds on TLC plate were detected under 270 nm. High-performance liquid chromatography (HPLC) was conducted using a semipreparative C18 reverse phase column (Symme-

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tryPrep C_{18} column, 7 μm, 7.8 × 300 mm, Waters, Milford, MA) in a Gilson HPLC system (Gilson, Middleton, WI). The separation of the antibiotic β-asarone was monitored at an absorbance of 270 nm by a UV−vis detector (118 UV−vis detector, 0.2 mm cell path, Gilson). The UV spectrum was recorded on a Beckman DU 650 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX 500 NMR spectrometer (Bruker, Rheinstetten, Germany) using CD_3OD with tetramethylsilane (TMS) as internal standard. Two-dimensional NMR spectra, such as ^{1}H−^{1}H COSY, ROSEY, HMQC, and HMBC, also were measured using the Bruker AMX 500 NMR spectrometer. The EI mass spectrum was measured on a JEOL JMS-700 MStation mass spectrometer (JEOL, Tokyo, Japan).

Isolation of Antifungal Compounds from Rhizomes of *A. gramineus*. The *A. gramineus* samples were purchased from Jingun Co. in Seoul, Korea. A voucher specimen has been deposited at the Laboratory of Molecular Plant Pathology, Korea University, Seoul, Korea. The rhizomes (1 kg) of *A. gramineus* were shade-dried. The powdered *A. gramineus* sample was extracted with 80% aqueous MeOH on a shaker for 5 days. The MeOH extract was filtered through Whatman no. 1 filter paper and concentrated in vacuo. The remaining residue was dissolved in distilled water at pH 5.0 and extracted twice with an equal volume of EtOAc at room temperature. The EtOAc layers were pooled and extracted with distilled water to remove the hydrophilic compounds. The resulting organic solvent layers were evaporated under reduced pressure. The concentrated EtOAc extract was subjected to flash column chromatography over silica gel ($60F_{254}$, 63−200 μm, Merck) with successive elutions with $CHCl_3$/MeOH in increasing order of polarity (90:10, 80:20, 70:30, 50:50, 30:70, 10:90, v/v). Each of the $CHCl_3$/MeOH eluates was concentrated and evaluated for antifungal activity. The active 90% $CHCl_3$/10% MeOH eluates that exhibited antifungal activity against test fungi were further purified on a silica gel column (150 × 200 mm) with a stepwise gradient of *n*-hexane and Me_2CO (100:0, 90:10, 80:20, 70:30, 50:50, 30:70, 10:90, v/v). A small volume of the concentrates of 100% *n*-hexane and 90% hexane/10% acetone eluates were separated by the gel filtration of the C26/100 column (Pharmacia) packed with Sephadex LH-20 resin using MeOH as a mobile phase at a 0.2 mL/min rate. The 2-mL fractions were collected using a fraction collector (Redifrac, Pharmacia). Each of all the fractions was bioassayed for antifungal activity against *Colletotrichum orbiculare* and *Magnaporthe grisea*. The active fractions 47−155 were subjected to preparative TLC on glass plates (20 × 20 × 0.2 mm) coated with silica gel ($60GF_{254}$, Merck). The TLC plates developed in the *n*-hexane/EtOAc (8:2) solvent system were visualized under UV light. The activity of bands on TLC plates was examined by bioautographic technique. Scraped active bands were collected and extracted with 100% MeOH at 28 °C in a shaking incubator for 24 h. The pooled extract was bioassayed against *C. orbiculare*. The concentrated extracts from antifungal-active TLC bands were further purified on Sephadex LH-20 column (10 × 950 mm) chromatography. The Sephadex LH-20 column was eluted with MeOH at a flow of 0.2 mL/min. Gel filtration was performed three times under the same conditions. The isolation of the antifungal compound β-asarone was performed by HPLC system on a symmetry Prep C_{18} column (7 μm, 7.8 × 300 mm, Waters) using a linear gradient solvent system from 50% CH_3CN in H_2O to 70% CH_3CN in H_2O at a flow rate of 2 mL/min. The separation was monitored at an absorbance of 270 nm by a UV−vis detector (118 UV−vis detector, 0.2 mm cell path, Gilson).

In Vitro Antimicrobial Activity of β-Asarone. Antifungal and antibacterial activities of the purified compound β-asarone against several plant pathogenic microorganisms were evaluated in 24-well microtiter plates (Cell wall, Corning Glass Works, Corning, NY). The plant pathogenic fungi and oomycete used in the bioassay were *Alternaria mali*, *Botrytis cinerea*, *Cladosporium cucumerinum*, *Colletotrichum orbiculare*, *Cylindrocarpon destructans*, *Fusarium oxysporum* f.sp. *lycopersici*, *Magnaporthe grisea*, *Phytophthora capsici*, *Pythium ultimum*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum*. They were incubated on potato dextrose agar at 20−28 °C. Spore and mycelial fragments were harvested and suspended at 10^6 spores/mL. Yeast (*Candida albicans* and *Saccharomyces cerevisiae*) and bacteria including *Bacillus subtilis*, *Erwinia carotovora* subsp. *carotovora*, *Ralstonia solanacearum*, and *Xanthomonas campestris* pv. *vesicatoria* also were evaluated for antimicrobial activity. Yeast and bacteria were cultured on a nutrient agar at 28 °C. Inoculum concentrations for the bioassay were adjusted to 10^4 spores/mL. Concentrations of the antifungal compound β-asarone were adjusted to 0, 0.1, 0.5, 1, 10, and 50 μg/mL. Ten microliters spore and mycelial suspensions was then inoculated in each well. The inoculated well plates were incubated at 28 °C. The inhibitory effects of the antibiotic β-asarone on the growth of test organisms were evaluated after incubation for 2−4 days. The lowest concentration of antibiotic that completely inhibited the growth of the microorganisms was considered a minimum inhibitory concentration (MIC).

Evaluation of in Vivo Control Efficacy of β-Asarone. The protective activity of the antibiotic β-asarone on cucumber plants against *Co. orbiculare* was evaluated in a growth room. Cucumber (*Cucumis sativus* L. cv. Baekrokdadaki) seeds were sown in a plastic pot (5 × 15 × 10 cm) containing steam-sterilized soil mix (peat moss, perlite, and vermiculite, 5:3:2, v/v/v), sand, and loam soil (1:1:1, v/v/v). Cucumber plants were raised in a growth room at 28 ± 2 °C with 80 μmol of photons/m^2/s for 16 h a day, The commercial fungicide chlorothalonil was used to compare the antifungal activity with that of the purified antibiotic β-asarone. Chlorothalonil and the purified sample dissolved in MeOH, respectively, were diluted with 0.1% Tween 20 to give the concentrations of 10, 50, 100, and 500 μg/mL. One day before inoculation with *Co. orbiculare,* each diluted chemical solution was sprayed on the primary leaf of cucumber plant at the three-leaf stage. Spore suspensions (10^6 spores/mL) of *Co. orbiculare* in 0.05% Tween 20 solution were uniformly sprayed on the leaves of cucumber plants. The inoculated cucumber plants were transferred into a dew chamber at 28 ± 1 °C. After incubation for 24 h, plants were placed in the growth room. Lesions on the secondary leaves of cucumber plants were counted 6 days after inoculation. The means of data at each concentration were separated using the least significance difference (LSD) at $P = 0.05$.

The ability of the purified compound β-asarone to suppress leaf blast development on rice was evaluated in a greenhouse. Rice (*Oryza sativa* L. cv. Nakdong) was raised in a plastic pot (5 × 15 × 10 cm) filled with the steam-sterilized soil. The antifungal activity of the commercial fungicide tricyclazole was compared with that of the antibiotic β-asarone. The antibiotic β-asarone and tricyclazole dissolved in MeOH, respectively, were diluted with 0.1% Tween 20 solution to give 1, 10, 50, 100, and 500 μg/mL. Each of the solutions was sprayed on the leaves of rice at the eight-leaf stage 1 day before inoculation with *M. grisea*. A conidial suspension (10^5 conidia/mL) of *M. grisea* was sprayed on the leaves of rice until runoff. The inoculated plants were placed in a dew chamber for 24 h at 28 °C and then transferred to the greenhouse. Lesions on the seventh leaves at the eight-leaf stage were counted 5 days after inoculation when typical lesions appeared on the leaves of plants. The means of all data at each concentration were separated using the LSD at $P = 0.05$. Statistical analysis of data was conducted using the Statistical Analysis Systems Institute Inc. procedure (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Extraction and Purification of the Antifungal Compound β-Asarone from Rhizomes of *A. gramineus*. During the screening process for antifungal plant materials, selected *A. gramineus* was extracted with 80% MeOH. The concentrated crude *A. gramineus* extracts were then partitioned with EtOAc. Antifungal activity against *Co. orbiculare*, *M. grisea*, and *B. cinerea* was detected in the EtOAc layer. The antifungal-active organic layers were pooled, concentrated, and further chromatographed on a silica gel (63−200 μm, Merk) column. The silica gel column was eluted with stepwise gradients of $CHCl_3$ and MeOH. The fractions eluted with $CHCl_3$ and MeOH (90:10, v/v) showed a high level of antifungal activity against *Co. orbiculare*, *M. grisea*, and *R. solani*. The antifungal-active

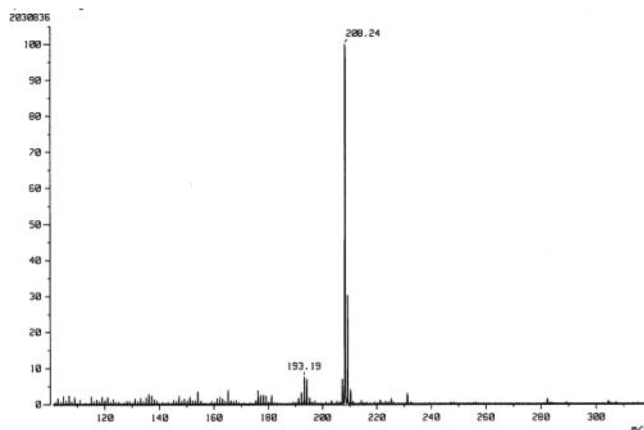

Figure 1. EI mass spectrum of the antibiotic β-asarone.

90% $CHCl_3$ fraction was rechromatographed on a silica gel column using a stepwise gradient elution system of *n*-hexane and Me_2CO. The 90 and 100% *n*-hexane fractions that were highly active against *Co. orbiculare*, *M. grisea*, and *R. solani* were further purified by Sephadex LH-20 column (26 × 950 mm) chromatography. Antifungal activity against *Co. orbiculare* and *M. grisea* was detected in fractions 46−149. The antifungal compounds of Sephadex LH-20 fractions active against *Co. orbiculare* and *M. grisea* were further purified by TLC. Silica gel plates loaded with the antifungal-active fractions were developed by a solvent system of *n*-hexane/EtOAc (8:2, v/v). MeOH elutes of the active band scraped from preparative TLC plates were bioassayed against *Co. orbiculare* using a paper disk method and then purified by gel filtration chromatography of Sephadex LH-20 (10 × 950 mm column). Antifungal activity was detected in fractions 111−171. The antifungal compound active against *Co. orbiculare* and *M. grisea* was detected by bioautography on a silica TLC plate. The clear inhibition zone occurred at the position of R_f 0.36 on the silica gel TLC plates developed by a solvent system of *n*-hexane/EtOAc (8:2, v/v). The HPLC profile of the antifungal compound β-asarone showed an antifungal active peak at the retention time of 15.86 min at 270 nm. Three hundred milligrams of the antifungal compound was yielded from the extract of *A. gramineus*.

Structure Elucidation of Antifungal Compound β-Asarone. The molecular formula $C_{12}H_{16}O_3$ of the antifungal compound β-asarone was established on the basis of the analysis of high-resolution EI mass spectral data showing a molecular ion peak at *m/z* 208.24 (M^+) (**Figure 1**). ^1H, ^{13}C, and DEPT NMR spectral data are shown in **Table 1**. Two singlet aromatic protons (δ 6.86 and 6.65), two olefinic protons (δ 6.42 and 5.68), three methoxyl protons (δ 3.85, 3.78, and 3.77), and methyl protons (δ 1.80) were confirmed by ^1H NMR spectral data. Twelve carbon signals observed in the ^{13}C NMR spectrum were assigned to four quaternary carbons at δ 153.4, 150.4, 143.7, and 119.6, three sp^2 methines at δ 126.1, 126.0, 116.4, and 99.4, three aromatic methoxyl carbons at δ 57.6, 56.9, and δ 56.7, and a methyl carbon at δ 14.9 by the DEPT spectrum. The structure of the antibiotic β-asarone was determined by the interpretation of $^1H-^1H$ COSY, NOESY, and HMBC spectra. In light of all the spectral data, the structure of the antibiotic compound was identified to be β-asarone, the *cis*-isomer of 2,4,5-trimethoxy-1-prophenylbenzene (**Figure 2**). The mass spectrometry and ^1H and ^{13}C NMR data were consistent with those previously reported for β-asarone (*10*, *13*).

Table 1. ^1H and ^{13}C NMR Spectral Data of the Antibiotic β-Asarone in CD_3OD

position	$^{\delta}H$	$^{\delta}C$
1	6.86 (1H, s)[a]	116.4 (CH)[b]
2		143.7 (C)
3		150.4 (C)
4	6.65 (1H, s)	99.4 (CH)
5		153.4 (C)
6		119.6 (C)
7	6.42 (1H, dq, *J* = 12.0, 2.0)	126.1 (CH)
8	5.68 (1H, dq, *J* = 12.0, 7.0)	126.0 (CH)
9	1.80 (3H, dd, *J* = 7.0, 2.0)	14.9 (CH_3)
10	3.77 (3H, s)	56.9 (CH_3)
11	3.85 (3H, s)	56.7 (CH_3)
12	3.78 (3H, s)	57.6 (CH_3)

[a] Proton resonance integral, multiplicity, and coupling constants (*J* = Hz). Abbreviations of signal multiplicity are as follows: s, singlet; dd, doublet of doublets; dq, doublet of quartets. [b] DEPT spectral data.

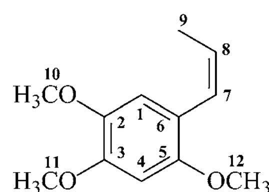

Figure 2. Structure of the antibiotic β-asarone isolated from *A. gramineus*.

α-Asarone, *trans*-2,4,5-trimethoxy-1-propenylbenzene, was earlier isolated from the *Guatteria guameri* plants growing in southern Mexico (*14*). β-Asarone, *cis*-2,4,5-trimethoxy-1-prophenylbenzene, was found in other related plant species including *Acorus calamus*. γ-Asarone, 1-allyl-2,4,5-trimethoxybenzene, is a rare phenylpropanoid first isolated from *Caesulia axillaries* and later detected as a biologically active constituent of various essential oils (*13*). Studies of structure−activity relationships of *trans*-asarone and related phenylpropanids against the green algae *Selenastrum capricornutum* revealed that their biological activity was more enhanced by an increase in the number in methoxy groups (*15*). The strongest biological activity of asarone was shown by the methoxy groups positioned ortho and para to the alkyl side chain (*15*). In contrast, the presence of a methoxy group in the meta position has been suggested to affect deterrent activity (*16*).

In Vitro and in Vivo Antifungal Activities of the Antifungal Compound β-Asarone. The minimum inhibitory concentrations (MIC) of the purified antibiotic β-asarone were evaluated by in vitro inhibition assay of mycelial growth on microtiter dishes (**Table 2**). The antibiotic β-asarone inhibited the mycelial growth of several plant pathogenic fungi such as *C. cucumerinum*, *Co. orbiculare*, and *M. grisea*. In particular, the growth of *P. ultimum* was inhibited even at a low concentration of 0.5 μg/mL. The growth of *B. subtilis*, *E. carotovora* subsp. *carotovora*, *R. solanacearum*, and *X. campestris* pv. *vesicatoria* was slightly suppressed by the antibiotic β-asarone, whereas β-asarone did not inhibit the growth of yeasts *C. albicans* and *S. cerevisiae*, even at the concentration of 100 μg/mL. Antimicrobial activities of asarone were earlier reported (*17*). Asarone exhibited inhibitory activity against *Escherichia coli* and *Bacillus subtilis* at 25 ppm. However, Momin and Nair (*18*) have recently demonstrated that no inhibitory activity against the bacteria *E. coli*, *Staphylococcus aureus*, and *Staphylococcus epidermis* was shown at 100 μg/mL of asarone. There also were no antifungal activities against *Aspergillus flavus*, *Aspergillus parasiticus*, and *Fusarium oxysporum* at

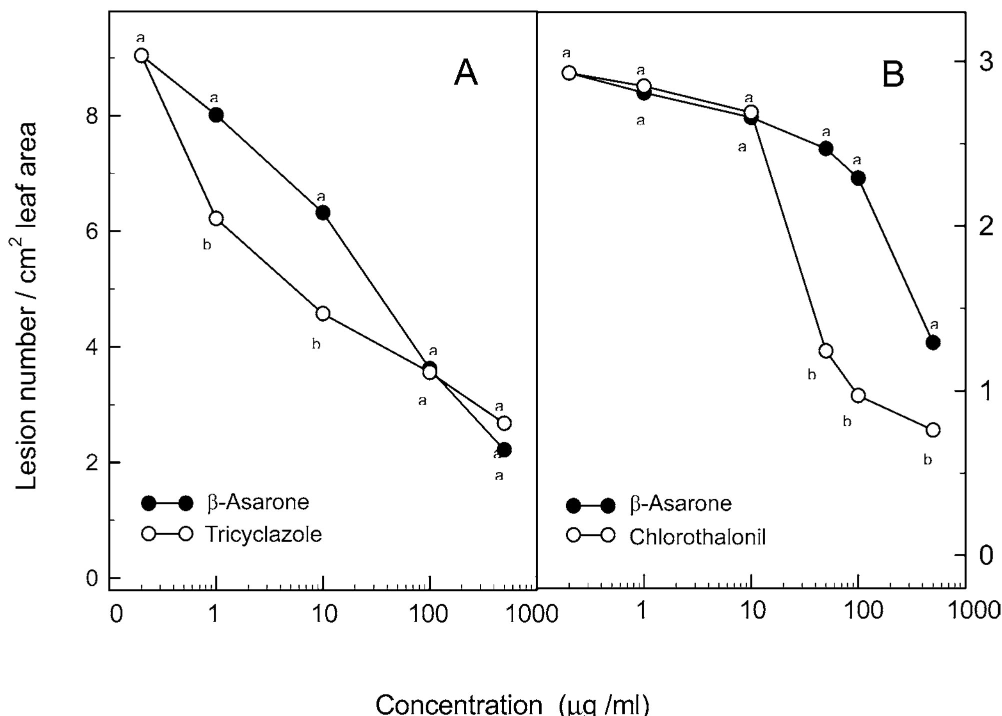

Figure 3. In vivo efficacies of the antibiotic β-asarone and the commercial fungicides tricyclazole and chlorothalonil for the control of (**A**) rice blast and (**B**) cucumber anthracnose diseases caused by *M. grisea* and *Co. orbiculare*, respectively. Solutions of each compound were sprayed on leaves 1 day before inoculation. Numbers of lesions on leaves were rated on day 6 after inoculation. Means at each concentration followed by the same letter are not significantly different ($P = 0.05$) according to the least significant difference test.

Table 2. Minimum Inhibitory Concentrations (MIC) against Growth of Various Microorganisms of the Antibiotic β-Asarone from Rhizome of *A. gramineus*

microorganism	MIC[a] (µg/mL)
Alternaria mali	>100[b]
Botrytis cinerea	>100
Cladosporium cucumerinum	30
Colletotrichum orbiculare	30
Cylindrocarpon destructans	>100
Fusarium oxysporum f.sp. *lycopersici*	>100
Magnaporthe grisea	30
Phytophthora capsici	>100
Pythium ultimum	0.5
Rhizoctonia solani	>100
Sclerotinia scleotiorum	>100
Bacillus subtilis	50
Candida albicans	>100
Erwinia carotovora subsp. *carotovora*	50
Ralstonia solanacearum	50
Saccharomyces cerevisiae	>100
Xanthomonas campestris pv. *vesicatoria*	50

[a] The lowest concentration that completely inhibits the growth of test microorganism was determined after incubation for 3–5 days. [b] >100 indicates that the growth of test microorganisms was not inhibited at the concentration of 100 µg/mL.

100 µg/mL. Slight inhibitory activity occurred against the growth of yeasts *C. albicans*, *C. parasilasis*, and *C. kruseii* at 100 µg/mL. In vivo efficacy of the antibiotic β-asarone for the control of leaf blast in rice plants was evaluated under the greenhouse condition (**Figure 3A**). As concentrations of the antibiotic β-asarone and the commercial fungicide tricyclazole increased, *M. grisea* infection was drastically inhibited on the rice leaves at the eight-leaf stage. Control efficacies of the β-asarone and tricyclazole were generally similar to each other at the concentrations of 100 and 500 µg/mL. The development of *Co. orbiculare* lesions on second leaves of cucumber plants was inhibited by the treatment with the antibiotic β-asarone and the commercial fungicides chlorothalonil (**Figure 3B**). At the two-leaf stage, treatment of cucumber leaves with 500 µg/mL of the antibiotic β-asarone greatly suppressed lesion formation. Chlorothalonil was more effective than the antibiotic β-asarone for the control of the anthracnose disease on cucumber leaves. The antibiotic β-asarone not only showed a strong inhibitory effect on the mycelial growth of *Co. orbiculare* but also suppressed anthracnose development on cucumber leaves. Although its in vivo fungicidal efficiency was somewhat less effective than that of the commercial fungicide chlorothalonil, the cucumber leaves were effectively protected from anthracnose infection by treatment with 500 µg/mL of the antibitic β-asarone. These results suggest that the antibiotic β-asarone of *A. gramineus* may be a good potent antifungal agent against some plant pathogens.

β-Asarone has been demonstrated to exhibit antigonadal activity potentially useful in insect control (*19*). More recently, insecticidal activity of asarones was found in *A. gramineus* rhizome (*20*). Asarones also showed a dual effect on the larvae of the nematode *Taxocara canis* (*21*). However, there is little information about the antifungal activity of the β-asarone from *A. gramineus* and its in vitro and in vivo antifungal activities against plant fungal pathogens *M. grisea* and *Co. orbiculare*.

Taken together, β-asarone from the *A. gramineus* in our study has not only a potent in vitro antifungal activity against several plant pathogenic fungi but also in vivo control efficacy against cucumber anthracnose and rice blast. However, β-asarone was demonstrated to have a carcinogenic effect in vivo (*22*), a mutagenic activity in vitro (*23*), and a spasmolytic effect (*24*) and was also shown to induce structural chromosome abnormalities in human lymphocytes in vitro (*11*). Accordingly, further research about safety would be necessary for the practical use of β-asarone.

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