JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 9483–9487 9483
DOI:10.1021/jf101694t

Antifungal Activity of Glyceollins Isolated from Soybean Elicited with *Aspergillus sojae*

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The aim of this study was to examine the efficacy of glyceollins isolated from soybean seeds elicited with *Aspergillus sojae* for its antifungal potential. Glyceollins I, II, and III were determined by HPLC-MS analysis. The glyceollins (200 and 600 μg/disk) revealed remarkable antifungal effect against *Fusarium oxysporum*, *Phytophthora capsici*, *Sclerotinia sclerotiorum*, and *Botrytis cinerea*, within the growth inhibition range of 10.9−61.0%, along with their respective MIC values ranging from 25 to 750 μg/mL. The glyceollins also had a strong detrimental effect on spore germination of all tested plant pathogens along with concentration- as well as time-dependent kinetic inhibition of *P. capsici*. Thus, the results obtained in this study demonstrate that glyceollins derived from soybean seeds elicited with *A. sojae* possess a wide range of fungicidal activity and could become an alternative to synthetic fungicides for controlling certain important fungal diseases.

KEYWORDS: Glyceollins; soybean; *Aspergillus sojae*; antifungal

INTRODUCTION

For many years, a variety of different chemical and synthetic compounds have been used as antimicrobial agents to inhibit plant pathogenic fungi. Antimicrobial chemicals such as benzimidazoles, aromatic hydrocarbons, and sterol biosynthesis inhibitors are often used to control plant disease in agriculture. However, there are a series of problems against the effective use of these chemicals in areas where the fungi have developed resistance (*1*). To overcome this problem, higher concentrations of these chemicals are used, but this increases the risk of high-level toxic residues in the products. Thus, there has been a growing interest in research for the possible use of plant-derived products, which can be relatively less damaging for pest and disease control in agriculture (*2*). In the past few years, due to concerns regarding the safety of synthetic antimicrobial agents, there has been an increase in naturally developed substances, which has resulted in a huge increase in the use of naturally derived compounds as potential antifungal agents. Also, plants have long been recognized to provide a potential source of chemical compounds or more commonly products known as phytochemicals (*3*).

Glyceollins, which are synthesized from daidzein in soybean cultured with fungi, have been shown to have antimicrobial effects and cancer preventive properties. Phytoalexins constitute a chemically heterogeneous group of low molecular weight antimicrobial compounds that are synthesized de novo and accumulate in plants in response to stress (*4*, *5*). The compounds are typically toxic to microbes but may not be to humans (*6*). In fact, phytoallexins found in grapes and herbal plants may be beneficial to human health, as seen in resveratrol, which has antioxidant, anti-inflammatory, and anticancer properties. Glyceollins represent another group of phytoalexins for which biosynthesis is increased in soybean in response to various stress signals such as fungal infection (*4*, *5*). As glyceollins have structures similar to those of other soybean isoflavonoids (**Figure 1**), there is a good possibility that they share common physiological properties. However, there is little report available on antifungal properties of glyceollins derived from soybean elicited with *Aspergillus sojae*.

In the current study, the antifungal properties of glyceollins isolated from soybean elicited with *A. sojae* were evaluated; it was found that the compounds inhibited the growth of some plant pathogenic fungi.

MATERIALS AND METHODS

Preparation of Glyceollin-Rich Extract. *A. sojae* cultures were grown at 25 °C in the dark on potato dextrose agar. Inocula were prepared by harvesting fungi after 5 days of incubation. Soybean seeds were surface-sterilized for 3 min in 70% ethanol, followed by a quick rinse with deionized water. Seeds were presoaked in sterile deionized water for 4 h before being placed into treatment chambers. The sample was moistened with distilled water. Soybean seeds were split into 8−10 particles in a food mixer (Hanil, Buchun, Korea). After they were placed into treatment chambers, an *A. sojae* spore suspension was applied to the cut surface of each seed.

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Published on Web 07/28/2010

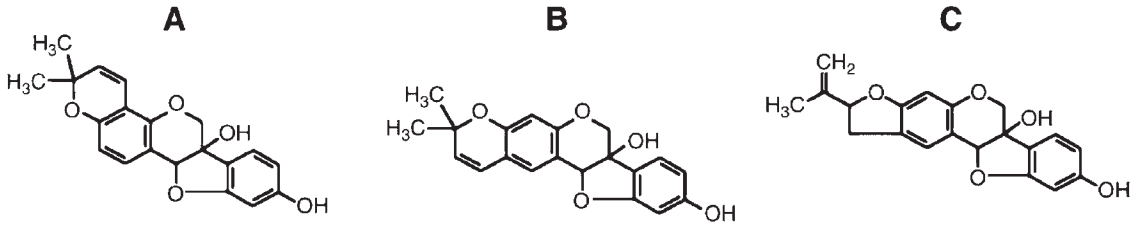

Figure 1. Molecular structures of glyceollins I (**A**), II (**B**), III (**C**).

The soybean seeds were stored in a dark chamber at 26 °C for 3 days. The soybean seeds infected with *A. sojae* (5 g) were homogenized in 80% aqueous ethanol (15 mL) and incubated at 50 °C for 1 h, cooled, then centrifuged at 14000*g* for 10 min. The extracts were filtered through a sterile syringe filter with a 0.45 μm pore size (Sartorius Biotech GmbH, Goettingen, Germany). The filtered extract was freeze-dried and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL prior to use.

Isolation of Glyceollins Using MPLC. The freeze-dried crude extract was suspended in H_2O and then successively partitioned with hexane and ethyl acetate (EtOAc) to distinguish between hexane-soluble and EtOAc-soluble fractions. The EtOAc-soluble fraction was subjected to silica gel column chromatography using EtOAc and MeOH mixtures (1:1) of increasing polarity (50:1 to 5:1) to yield five fractions (F1−F5). Fraction F2 was subjected to TLC and HPLC to confirm the existence of glyceollins, and then the fraction was evaporated under reduced pressure.

Analysis of Glyceollins Using HPLC−Mass Spectrometry. HPLC analyses were performed on a Jasco System Controller combined with a Jasco UV-2077 detector and PU-1580 pump as described previously (*7*). Isoflavones extracted from the soybean were directly analyzed by HPLC. Isoflavonoids and glyceollins were monitored at the wavelength of 280 nm. Glyceollins were identified using 90% pure standard, which we received from S. M. Boué at Tulane University. Separations were carried out using a Gemini C18 (150 × 2.0 mm; 5 μm; Phenomenex, Torrance, CA) reverse phase column. Elution was carried out at a flow rate of 0.8 mL/min with the following solvent system: A = 0.1% acetic acid/water; B = acetonitrile; from 10 to 35% B in 40 min, then from 35 to 10% B in 5 min followed by holding at 10% B for 10 min. Retention times for the isoflavonoids were as follows: daidzin, 6.7 min; genistin, 10.9 min; malonyldaidzin (MGD), 14.0 min; daidzein, 17.8 min; malonylgenistin (MGG), 18.4 min; genistein, 24.8 min; glyceollin III, 35.1 min; and glyceollins II/I, 36.0 min. All HPLC analyses were run in triplicate. The mass spectrometer used (Varian, Inc., Palo Alto, CA) was an ion trap equipped with a heated capillary electrospray interface. A positive ion mode was used with a sprayer needle voltage of 5000 V. The drying gas temperature was 300 °C.

Fungal Pathogens. The plant pathogenic fungi tested were obtained from the Korean Agricultural Culture Collection (KACC), Suwon, Republic of Korea. Cultures of each fungal species were maintained on potato dextrose agar (PDA) slants and stored at 4 °C. The fungal species used in the experiment were *Botrytis cinerea* KACC 40573, *Fusarium oxysporum* KACC 41083, *Sclerotinia sclerotiorum* KACC 41065, and *Phytophthora capsici* KACC 40157.

Preparation of Spore Suspension and Test Samples. The spore suspensions of *B. cinerea*, *F. oxysporum*, *S. sclerotiorum*, and *P. capsici* were obtained from their respective 10-day-old cultures and mixed with sterile distilled water to obtain a homogeneous spore suspension of 1×10^8 spores/mL. Glyceollins isolated from soybean elicited with *A. sojae* were dissolved in DMSO, and the sample was further diluted to prepare test samples; the final concentration of the solution was 40 mg/mL.

Antifungal Activity Assay. Petri dishes (9 cm diameter) containing 20 mL of PDA were used for antifungal activity assay, performed on solid media with the disk diffusion method (*8*). Sterile Whatman paper disks were loaded with 5 μL (200 g/disk) and 15 μL (600 g/disk) glyceollins, separately. The disks were dried and placed on the agar plates equidistantly. An agar plug of each fungal inoculum (6 mm diameter), removed from previous culture of each fungal pathogen, was placed upside down in the center of each Petri dish. Negative controls were prepared in the same solvents used to dissolve the test samples. The plates were incubated at 25 °C for 4−6 days, until the growth in the control plates reached the edges of the plates. Growth inhibition of each fungal strain was calculated as the percentage of inhibition of radial growth relative to the control. The plates were tested in triplicate for each treatment. The relative growth inhibition of treatment compared to control was calculated by percentage, using the following formula:

$$\text{inhibition (\%)} = [1 - ((\text{radial growth of treatment (mm)} / \text{radial growth of control (mm)})] \times 100$$

Determination of Minimum Inhibitory Concentration (MIC). The MICs were determined by the 2-fold dilution method (*9*) against *B. cinerea*, *F. oxysporum*, *S. sclerotiorum*, and *P. capsici*. A test sample was dissolved in DMSO. These solutions were serially diluted and were added to potato dextrose broth (PDB) to final concentrations of 10, 25, 50, 100, 250, 500, and 750 μg/mL, respectively. A 10 μL spore suspension of each test strain was inoculated in the test tube in a PDB medium and incubated for 2−4 days at 28 °C. The control tubes containing PDB medium were inoculated only with the fungal spore suspension. The minimum concentrations at which no visible growth was observed were defined as the MICs, which were expressed in micrograms per milliliter.

Spore Germination and Growth Kinetics Assay. Six concentrations of glyceollins (10, 25, 50, 100, 250, and 500 μg/mL) and one control (1% DMSO with sterile distilled water) were separately tested for the spore germination of different fungi including *B. cinerea*, *F. oxysporum*, *S. sclerotiorum*, and *P. capsici* (*10*). The samples were inoculated with the spore suspension of each fungal pathogen containing 1.0×10^8 spores/mL. From this, aliquots of 100 μL of spore suspension from each were placed on separate glass slides in triplicate. Slides containing the spores were incubated in a moisture chamber at 25 °C for 24 h. Each slide was then fixed in lactophenol-cotton blue and observed under the microscope for spore germination. About 200 spores were counted. The spores generating germ tubes were enumerated. The control DMSO (1%) was tested separately for the spore germination of different fungi. The percentage of spore germination inhibition was calculated by using the following formula:

$$\text{\% spore germination} = [\text{treatment (germinated spore/total spore)} / \text{control (germinated spore/total spore)} \times 100$$

P. capsici appeared to be more resistant than *F. solani* to the glyceollins. *F. solani* in the spore germination study was chosen as a test fungus for kinetic study and evaluation of the antifungal properties of glyceollins. The spore suspension (10 μL) of this fungal species was inoculated to different concentrations of glyceollins (25, 50, and 100 μg/mL) in a test tube and a homogeneous suspension (about 2×10^5 spore/mL) was made by inverting the test tubes three to four times. After specific intervals, namely, 30, 60, 90, 120, and 150 min, the reaction mixtures were filtered through a Whatman no. 1 filter paper and the retained spores were washed two or three times with sterile distilled water. The filter was then removed, and the spores were washed off into 10 mL of sterile distilled water. From this, 100 μL of spore suspension was placed onto a glass slide and incubated at 25 °C for 24 h. About 200 spores were counted, and the percentage of spore germination was calculated. Control sets were prepared with 1% DMSO with sterile distilled water. All experiments were conducted in triplicate.

RESULTS

Identification of Glyceollins by HPLC-MS. The changes in isoflavone and glyceollin concentrations were investigated in crude extracts from soybean seeds inoculated with species of *Aspergillus*. The HPLC profile of uninoculated soybean tissue was different from that of *A. sojae*-inoculated soybean tissue (**Figure 2A**). As

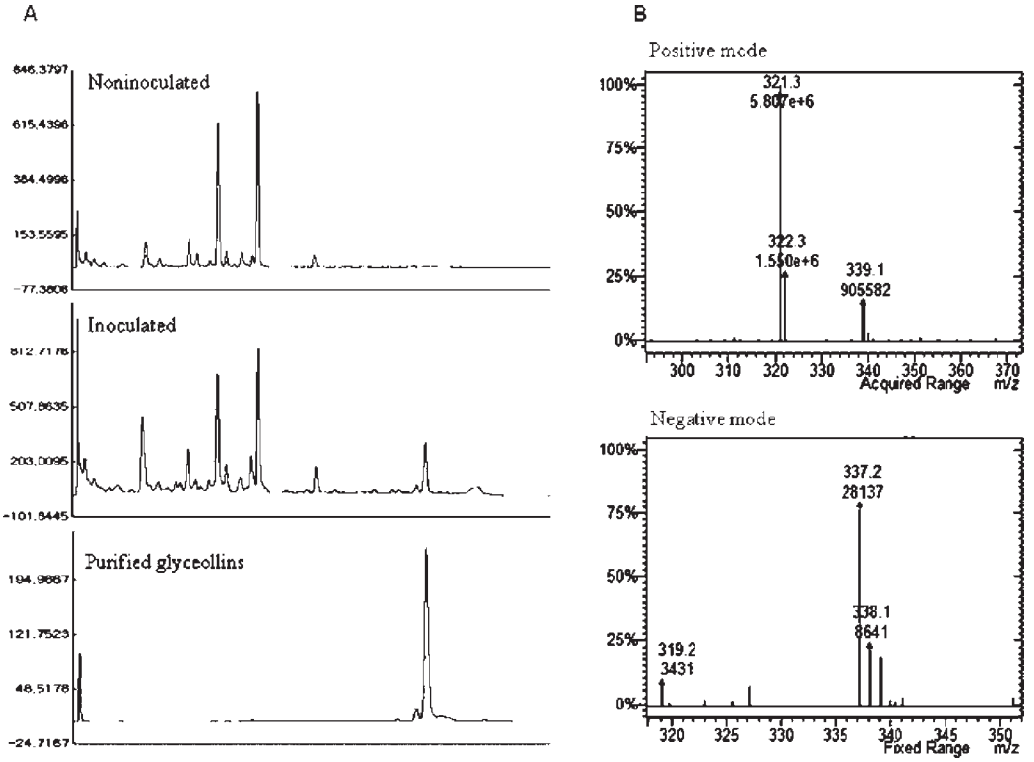

Figure 2. Isolation and identification of glyceollin production by HPLC and electrospray ionization−MS analysis. HPLC chromatograms show the comparison of control with fungus-inoculated soybean seed extract (**A**). Panel **B** shows positive and negative ion electrospray ionization mass spectra of glyceollin I isolated from extract of soybean seed infected with *A. sojae*.

Table 1. Antifungal Activity of Glyceollins Derived from Soybean Seed Elicited with *Aspergillus sojae*

	mycelial growth inhibition[a]			
fungal strain	mg/disk	mm	%	MIC[b] (μg/mL)
Fusarium oxysporum KACC 41092	0.2	24.3 ± 1.0 (33.5 ± 0.7)	27.4 ± 1.2	250
	0.6	20.0 ± 0.8 (33.4 ± 0.9)	40.1 ± 1.6	
Phytophthora capsici KACC 41092	0.2	24.2 ± 1.4 (38.7 ± 0.8)	37.5 ± 1.1	25
	0.6	22.1 ± 1.0 (37.1 ± 1.0)	40.5 ± 1.3	
Sclerotinia sclerotiorum KACC 41065	0.2	21.2 ± 0.7 (40.9 ± 0.4)	48.2 ± 0.8	50
	0.6	15.7 ± 1.1 (40.3 ± 0.6)	61.0 ± 1.1	
Botrytis cinerea KACC 40573	0.2	38.3 ± 1.5 (42.9 ± 0.7)	10.9 ± 0.7	750
	0.6	34.1 ± 1.3 (43.8 ± 0.3)	22.1 ± 0.9	

[a] Values are given as mean ± SD. of three experiments. mm, radial growth with sample (radial growth without sample); %, percentage of radial inhibition. [b] Minimum inhibitory concentration.

shown in **Figure 2A**, glyceollins I−III in the inoculated soybean were detected at higher concentrations than in the uninoculated soybean. The predominant isomer of glyceollins synthesized in cotyledon tissue cultured with *A. sojae* was glyceollin I, as identified by positive and negative ion electrospray ionization MS (**Figure 2B**). The molecular ions at $[M + H]^+$ m/z 339 (positive mode data) and at $[M - H]^+$ m/z 337 (negative mode data) represent the protonated glyceollins (MW 338), whereas the ions at $[M - H_2O + H]$ m/z 321 (positive mode data) and at $[M - H_2O - H]^+$ m/z 319 (negative mode data) detail the loss of one water molecule.

Antifungal Activity and Determination of MIC. The glyceollins exhibited a moderate to high antifungal activity against all of the plant pathogens tested. As shown in **Table 1**, glyceollins (200 and 600 μg/disk) showed potent inhibitory effect on the growth of *F. oxysporum* (27.4 and 40.1%), *P. capsici* (37.5 and 40.5%), *S. sclerotiorum* (48.2 and 61.0%), and *B. cinerea* (10.9 and 22.1%). *P. capsici* and *S. sclerotiorum* were found to be the most inhibited fungal pathogens by the glyceollins. A limited antifungal effect of glyceollins was observed against *B. cinerea*.

The MICs, defined as the lowest concentrations of glyceollins that resulted in complete growth inhibition of *F. oxysporum*, *P. capsici*, *S. sclerotiorum*, and *B. cinerea*, were found to be 250, 25, 50, and 750 μg/mL, respectively (**Table 1**). *P. capsici* and *S. sclerotiorum* were the most susceptible to the glyceollins. The control, a solvent, did not affect the growth of the sample strains at the concentrations used in this study.

Spore Germination and Growth Kinetics Assay. The inhibitory activity of glyceollins against spore germination of the test fungi is shown in **Figure 3**. DMSO (1%, v/v) as a control did not inhibit the spore germination of the plant pathogens tested. Glyceollins caused a dose-dependent inhibtion of fungal spore germination

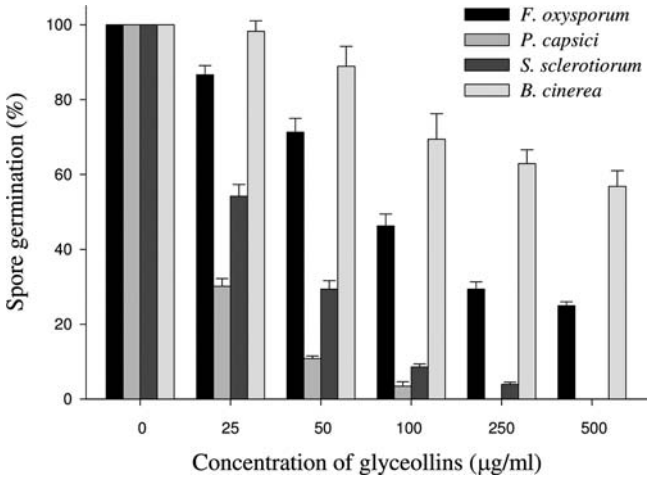

Figure 3. Effect of different concentrations of glyceollins on spore germination of tested fungi.

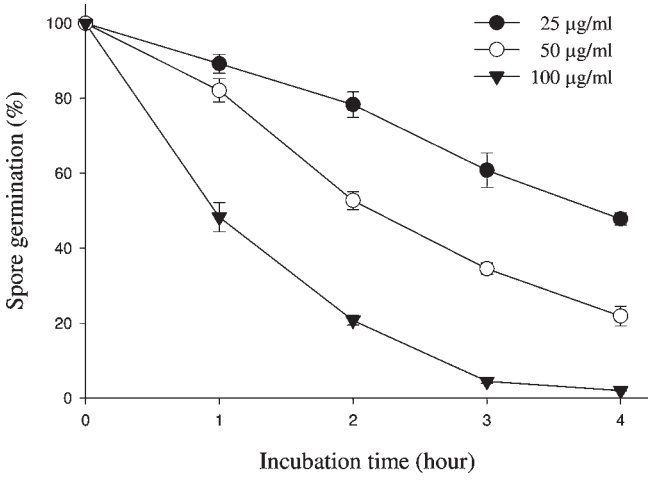

Figure 4. Kinetics of inhibition of *P. capsici* spore by glyceollins.

and a significant inhibition of fungal spore germination at different concentrations of glyceollins. A 100% inhibition of fungal spore germination was observed in *P. capsici* and *S. sclerotiorum* at 100, 250, and 500 μg/mL concentrations of glyceollins, respectively. Glyceollins also exhibited a potent inhibitory effect on the spore germination of *F. oxysporum* and *B. cinerea* with 40−70% growth inhibition at concentrations ranging from 100 to 500 μg/mL.

The antifungal kinetics of glyceollins against *P. capsici* is shown in **Figure 4**. Exposure of *P. capsici* spores to glyceollins for a period of 1−4 h caused a dose- and time-dependent inhibition of spore germination. The compounds at 25 μg/mL showed antifungal activity, with approximately 50% inhibition at the exposure time of 4 h. However, there was a marked increase in the killing rate at 100 μg/mL of glyceollins after 2 h of exposure and after 3 h of exposure with 95 and 100% inhibitions of spore germination, respectively. At low concentrations, a significant rate of inhibition was the characteristic feature of the glyceollins.

The inhibition activity of glyceollins against spore germination markedly increased at 100 μg/mL, showing 100% inhibition at 3 h of exposure,. Overall, glyceollins were found to be effective in the suppression of spore germination.

DISCUSSION

Increasing social and economic problems caused by fungi means there is a constant demand to produce safer food crops and to develop new antifungal agents. In general, plant-derived compounds are considered as nonphytotoxic and potentially effective against pathogenic fungi (*11*). In recent years, interest has been generated in the development of safer antifungal agents such as plant-based essential oils and extracts to control phytopathogens in agriculture (*2*). Historically, many plant -derived compounds have been reported to have antimicrobial properties (*12*, *13*). In particular, it is important to investigate the plants used in traditional medicines as potential sources for novel antimicrobial compounds (*14*). Also, the resurgence of interest in the natural control of plant pathogens and increasing consumer demand for effective, safe, natural products mean that quantitative data on plant-derived compounds are required. Various publications have documented the antimicrobial and antifungal activities of compounds derived from plants including rosemary, peppermint, bay, basil, tea tree, celery seed, and fennel (*15*−*17*). In general, because glyceollins are prenylated isoflavone compounds, which are natural classes of phytochemicals, it would seem reasonable that their antimicrobial or antifungal mode of action might be related to the unique structure of these isoflavone compounds.

Glyceollins, isolated from soybean elicited with *A. sojae*, showed remarkable antifungal effects against all of the plant pathogens tested in this study. This research also showed that glyceollins had a suppressive activity on fungal spore germination as well as a wide range of antifungal properties. The kinetic study of *P. capsici* showed that the exposure time of glyceollins had little effect on the fungicidal activity at lower concentrationas such as 25 μg/mL. In contrast, high doses of glyceollins including 50 and 100 μg/mL caused a much stronger inhibition on spore formation of *P. capsici*. This activity could be attributed to its coumestrolic structure. On the other hand, one of the fungal pathogens, *B. cinerea*, was found to be slighthy resistant to the compounds at a 0.6 mg/mL concentration with 22.1% of fungal mycelia growth inhibition.

Earlier papers on the antifungal properties of plant material of some species have shown to have various degrees of growth inhibitory effects against some *Fusarium*, *Botrytis*, and *Rhizoctonia* species depending on their chemical compositions (*18*−*20*). Some plant materials, such as wintergreen, eucalyptus, clove, and sage, have been researched extensively and reported to exhibit toxic and irritant properties (*13*, *21*, *22*). Despite these unfriendly traits, most of these plant materials are attractive sources for pharmaceutical or cosmetic products, indicating that toxic properties do not hamper their use (*23*, *24*). However, these should be subjected to safety evaluation as toxic or irritant compounds if they are to be considered for any new products for human use. Certain plant extracts and phytochemicals act in many ways on various types of disease complexes and may be applied to the crop in the same way as other agricultural chemicals. In the current study, pure compounds derived from soybean isoflavones showed antifungal activities against various pathogenic fungi. It would also be interesting to study the effects of plant-derived materials elicited by *A. sojae* on medically important fungi for development of new antifungal agents for the treatment of serious fungal diseases and infections in animals and humans along with plant fungal diseases.

In conclusion, this study indicated that prenylated isoflavone glyceollins, isolated from soybean elicited with *A. sojae*, displayed potential antifungal activity controlling the in vitro growth of certain important plant pathogenic fungi. On the basis of the above findings, it can be concluded that glyceollin compounds hold potential significance as natural alternatives to synthetic fungicides for use in agro-industries to control agricultural plant pathogenic fungi. However, to further confirm their applicable efficacy as natural fungicides, studies on their toxicological properties and issues of safety and environmental impact are still needed.

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Received for review May 4, 2010. Revised manuscript received June 26, 2010. Accepted June 30, 2010. This study was supported by a grant from the Agricultural R&D Promotion Center funded by the Korean government (108046-3), Republic of Korea.