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Screening and Evaluation of Antibacterial Metabolites from Entomopathogenic Fungi

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Abstract

Entomopathogenic fungal species have been investigated for their potential use as biological control agents owing to their natural role as insect pathogens. These fungi produce a wide range of secondary metabolites with high therapeutic values, such as antibiotics and cytotoxic substances. To evaluate the antibacterial activity of entomopathogenic fungi, 10 isolates from Korean soil were selected and tested for their activity against *Escherichia coli* by using fungal culture filtrates. Antibacterial activity was assessed using a two-step process: (1) a screening assay for the selection of fungal isolates and (2) a quantitative assay to evaluate the activity of select fungi. Although 4 fungal isolates were selected through the screening assay, only 3 fungal isolates, from *Beauveria bassiana* and *Metarhizium anisopliae*, showed high antibacterial activity according to the quantitative assay. The antibacterial activity of selected fungal culture filtrates was stable when exposed to heat and proteolytic enzyme treatments, which indicated that the antibacterial compound is not a protein. These entomopathogenic fungal metabolites might be useful as a source for bacterial control and in the pharmaceutical industry.

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Introduction

Entomopathogenic fungi are natural pathogens of insects and contribute to the regulation of host insect populations in the natural environment. Their mode of action against insects involves the attachment of conidia to the insect cuticle followed by germination, cuticle penetration, and internal dissemination throughout the insect (Vega *et al*., 2012). During this process, secreted enzymes, protein toxins, and secondary metabolites can be used by fungi to overcome the host immune system, modify host behavior, and defend host resources against competing pathogens and saprophytes (Isaka *et al*., 2005; Molnár *et al*., 2010).

Recently, secondary metabolites isolated from entomopathogenic fungi have been reported as potential bioactive substances (Isaka *et al*., 2005; Molnár *et al*., 2010). The antimicrobial effect of entomopathogenic fungi [e.g., *Beauveria bassiana* (Bals.) Vuill., *Metarhizium anisopliae* (Metchn.) Sorokin, *Isaria* spp., and *Lecanicillium* spp.] in the suppression of plant disease caused by plant pathogens such as *Alternaria solani*, *Botrytis cinerea*, *Fusarium oxysporum*, *Rizoctonia solani*,

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and *Pythium myriotylum* have been reported by numerous studies (Goettel *et al*., 2008; Lee *et al*., 2005). Additionally, these metabolites exhibit a wide variety of insecticidal, antibacterial, antifungal, anticancer, antioxidant, and antiviral activities, and they have been suggested as potential candidates for the development of new bioactive agents (Isaka *et al*., 2005; Pedras *et al*., 2002; Wang and Xu, 2012).

These findings confirm the potential of entomopathogenic fungi as sources of lead compounds in pharmaceutical interests. Therefore, in this study, we report our results by assessing the ability of entomopathogenic fungi isolated in Korea (Shin *et al*., 2013) to produce antibacterial metabolites against *Escherichia coli* and their biochemical characteristics.

Materials and Methods

Entomopathogenic fungal metabolites

Entomopathogenic fungi, which exhibit virulence against the great wax moth (*Galleria mellonella* L. [Lepidoptera: Pyralidae]), were used in this study (Table 1) (Shin *et al*., 2013). For initial screening of antibacterial compounds, 1 ml of sabouraud dextrose broth containing a yeast extract medium (SDYB; 10 g Bacto Peptone, 40 g Dextrose, and 10 g yeast extract in 1000 ml distilled water; pH 6.0) was placed in a 2-ml microcentrifuge tube. The samples were then inoculated with a one-agar block (6 mm) of fungi collected from 2-week-old potato dextrose agar (PDA; Difco, USA). Samples were cultured at 25°C in the dark and were shaken at 150 rpm for 10 days, after which the samples were centrifuged at 13,000 rpm for 10 min. The aqueous upper layer was transferred into a Plasmid DNA collection kit column (COSMO GENETECH) to filter spores and mycelia.

For quantitative assay, all fungal conidia were obtained by scraping a 2-week-old PDA plate and suspended in a 0.05% Tween-80 solution. The conidial suspension was vigorously agitated and filtered through cotton to remove mycelial debris. After counting the number of conidia, 50 µl of the conidial suspension (2×10^6 conidia/ml) was inoculated with 20 ml of SDYB medium in a 100-ml Erlenmeyer flask and cultured using the abovementioned method. After 10 days, the samples were centrifuged at 13,000 rpm for 20 min. The pellet was removed and the supernatant was filtered using a 0.45 µm membrane filter (ADVACTEC No. 2) to separate the crude extract from the mycelium and spore mass. All fungal cell-free culture filtrates were stored at -70°C until antibacterial activity could be detected.

Table 1. Entomopathogenic fungal isolates used in this study

Species	Isolate
Beauveria bassiana	
	CN5R1W1
	CB12M1W1
	JN1T1W1
Isaria farinosa	
	KB20M2W1
	KB22G1W1
Metarhizium anisopliae var. *anisopliae*	
	JB5G2W1
	CN6S1W1
	CN2T1W1
	KW5M4W1
Myrothecium sp.	
	JB11S2W2

Bacterial strain

For the preparation of bacteria, *E. coli* XL1Blue was cultured using a LB media (10 g tryptone, 5 g yeast extract, and 5 g sodium chloride in 1000 ml distilled water; pH 7.0). One ml of *E. coli* culture suspension incubated overnight was inoculated into 100 ml of LB media and grown to the mid-exponential phase ($O.D._{650\ nm} = \sim 0.5$).

Antibacterial activity assay

Antibacterial activity against *E. coli* was determined using a broth dilution method (du Toit and Rautenbach, 2000). The bacterial cells of the mid-exponential phase were diluted with LB media to 1×10^3 CFU/ml (for the screening assay) and 5×10^5 CFU/ml (for the quantitative assay), respectively. For the screening assay, 100 µl of bacterial suspensions were placed in 96 wells with 100 µl of the fungal culture filtrate and incubated at 37°C for 16 h. To determine bacterial growth inhibition, the absorbance of the each well was measured at 650 nm by using a

microplate reader (Molecular Devices, UK). A bacterial culture well grown in SDYB or LB medium without the fungal culture filtrate was used as the positive control well.

For the quantitative assay, 100 µl of bacterial suspensions were placed in 96 wells and then mixed with 100 µl of the fungal culture filtrate at different concentrations (i.e., 1%, 10%, 20%, 40%, 60%, 80%, and 100%). The fungal culture filtrate was diluted with SDYB media. For the control, bacterial suspensions were cultured in SDYB media with different concentrations of PBS in place of the fungal culture filtrate. All experiments were performed in triplicate.

MTT assay

Bacterial cell viability was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay based on the reduction of MTT to formazan dye by active mitochondria. Briefly, 20 µl of the MTT solution (5 mg/ml in PBS) was added to each well and incubated for 4 h. Then, the MTT-spiked media was carefully removed by centrifugation at 13,000 rpm for 10 min, and formazan formed in the cells was dissolved in 200 µl of dimethyl sulfoxide. Absorbance at 540 nm was read using a micro plate reader (Molecular Devices, UK).

Stability of antibacterial activity of culture filtrate

For determining the heat stability, the culture filtrate was treated at various temperatures (50°C, 80°C, 100°C, and 121°C) for 15 min. After thermal shock, the culture filtrate was quickly cooled to 25°C, and its antibacterial activity was evaluated.

To test its stability in the presence of a protease, the culture filtrate was treated with proteinase K (Sigma, USA) at a final concentration of 1 mg/ml under the condition of 37°C for 2 h. Then, the culture filtrate was autoclaved at 121°C for 15 min to inactivate the enzymes before the antibacterial activity assay was conducted.

Results

Screening of fungi showing antibacterial activity

Primary screening for the antibacterial activity of entomopathogenic fungal isolates was carried out using a broth dilution assay. Among the 10 isolates tested, the culture filtrate of 4 isolates (i.e., *B. bassiana* CN5R1W1, *M. anisopliae* var. *anisopliae* JB5G2W1, CN6S1W1, and *Myrothecium* sp. JB11S2W2) exhibited high antibacterial activity (Fig. 1). Bacterial growth was reduced to approximately 2–20 and 3–30 times that in the LB and SDYB media, respectively. These fungal isolates were used for further studies to clarify the production of antibacterial compounds.

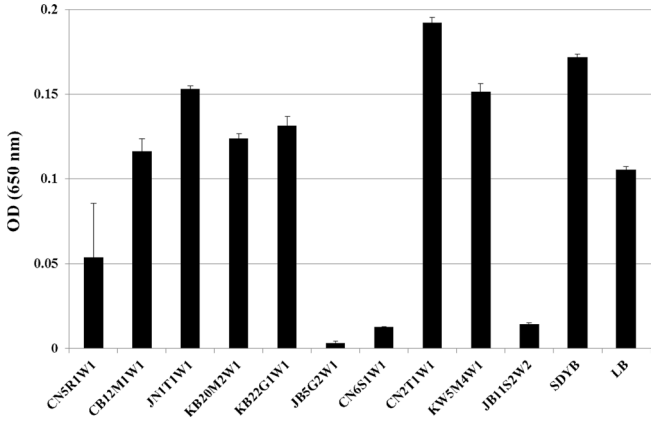

Fig. 1. Growth of *E. coli* in LB media with culture filtrates of entomopathogenic fungi for 16 h. Vertical bars correspond to standard error.

Quantitative assay of antibacterial activity

Fungi were selected for scale-up culture with the same concentration of conidia and they were them subjected to a quantitative assay. The culture filtrate of *M. anisopliae* var. *anisopliae* JB5G2W1 showed the highest antibacterial activity, followed by that of *M. anisopliae* var. *anisopliae* CN6S1W1 and *B. bassiana* CN5R1W1 (Fig. 2). Antibacterial activity increased with increasing concentrations of the culture filtrate. However, the culture filtrate of *Myrothecium* sp. JB11S2W2, which showed a high activity rate at the initial screening stage, did not show any significant antibacterial activity in the quantitative assay.

Cell viability assay

Surviving bacterial cell numbers in each well treated with 100% of the culture filtrate were determined indirectly by MTT

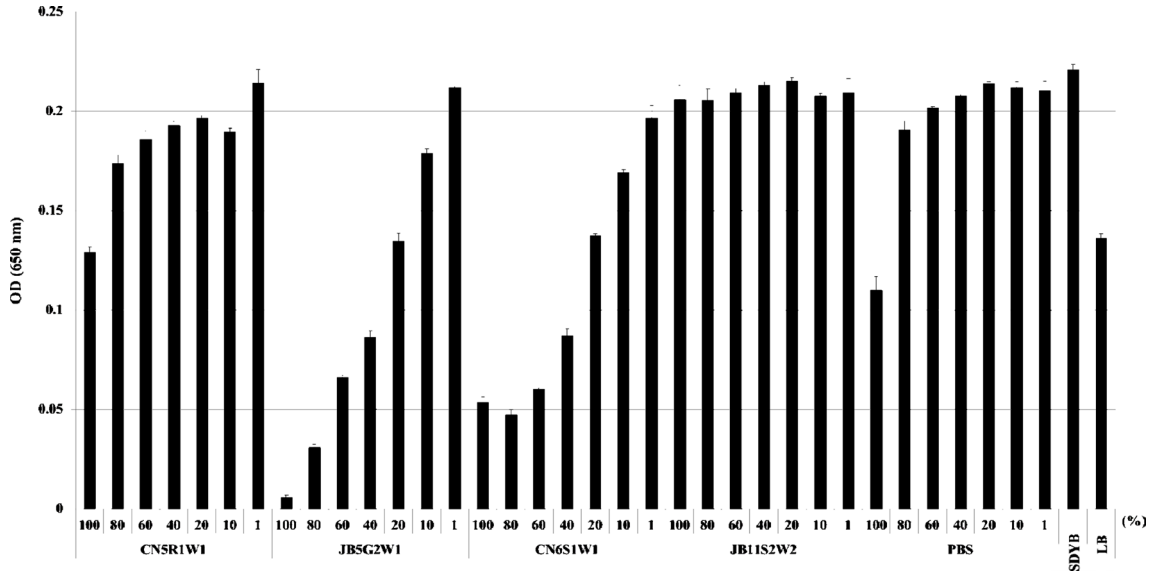

Fig. 2. Antibacterial activity of culture filtrates of entomopathogenic fungi at different concentrations (1%, 10%, 20%, 40%, 60%, 80%, and 100%) against *E. coli* for 16 h. Vertical bars correspond to standard error.

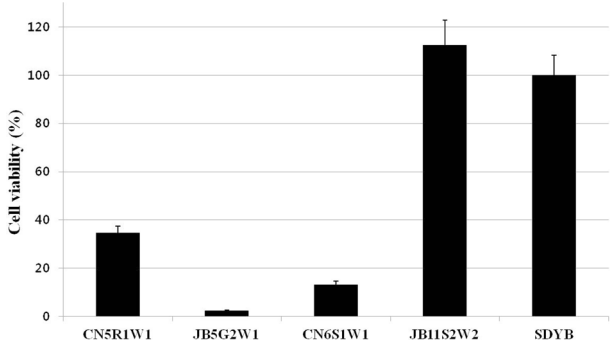

Fig. 3. Bacterial cell viability for fungal culture filtrates. Cell viability was assessed by MTT assay. Vertical bars correspond to standard error.

assay. As a result, for all fungal culture filtrates, no differences were detected between the O.D. (Fig. 2) and cell viability values (Fig. 3) obtained by both assays.

Stability of antibacterial activity

The stability of culture filtrates to heat and protease treatments were evaluated for antibacterial activity. Heat treatment did not influence the antibacterial activity of the culture filtrate for all tested isolates (Table 2). Furthermore, the culture filtrate retained its antibacterial activity even after treatment at 121°C for 15 min. The hydrolytic enzyme, proteinase K, also had no effect on the antibacterial activity of all culture filtrates (Table 2).

Discussion

Beauveria bassiana and *M. anisopliae* are the most commonly isolated species in the world (Vega *et al*., 2012), thus making them the most widely known among entomopathogenic fungi and fostering their use as biocontrol agents against a variety of pests in various countries (de Faria and Wraight, 2007). These 2 species are also the most commonly used in the study of fungal metabolites (Pedras *et al*., 2002; Wang and Xu, 2012). In the present study, *B. bassiana* and *M. anisopliae* (including *Isaria farinosa* and *Myrothecium* sp.) culture filtrates were tested for their antibacterial activity against *E. coli*. Initial screening results showed that 4 of 10 isolates exhibited high antibacterial activity against bacterial cells (1×10^2 CFU). These results corresponded with previous reports that entomopathogenic fungi can exhibit antibacterial activity (Isaka *et al*., 2005; Pedras *et al*., 2002; Wang and Xu, 2012). However, *Myrothecium* sp. JB11S2W2 showing high antibacterial activity in the initial screening assay did not show any antibacterial activity against a high concentration of bacterial cells (i.e., 5×10^4 CFU; Fig. 2). This result may be explained by 2 lines of reasoning. First, the data obtained may be because of differences in the antibacterial compound yield between initial screening and quantitative assays. In the initial screening assay, the concentration of conidia for inoculation

Table 2. Effect of temperature and enzymatic shock on the antibacterial activity of culture filtrates of entomopathogenic fungi. Data represent mean values of 3 replicates with standard error

Isolates	Treatment	OD (650 nm)
CN5R1W1		
	50°C, 15 min	0.12 ± 0.000
	80°C, 15 min	0.075 ± 0.000
	100°C, 15 min	0.073 ± 0.002
	121°C, 15 min	0.085 ± 0.004
	Proteinase K	0.099 ± 0.000
	Untreated	0.129 ± 0.002
JB5G2W1		
	50°C, 15 min	0.004 ± 0.000
	80°C, 15 min	0.001 ± 0.000
	100°C, 15 min	0.001 ± 0.000
	121°C, 15 min	0.000 ± 0.000
	Proteinase K	0.000 ± 0.000
	Untreated	0.006 ± 0.000
CN6S1W1		
	50°C, 15 min	0.057 ± 0.001
	80°C, 15 min	0.023 ± 0.005
	100°C, 15 min	0.038 ± 0.001
	121°C, 15 min	0.041 ± 0.001
	Proteinase K	0.047 ± 0.001
	Untreated	0.053 ± 0.002

was not determined, but it was determined and inoculated in the quantitative assay. The conidial concentration for inoculation may have been insufficient to produce a substantial yield of antibacterial compounds from *Myrothecium* sp. JB11S2W2. Second, it may be related to differences in the tested bacterial concentrations. Although antibacterial compound(s) from *Myrothecium* sp. JB11S2W2 was sufficient to reduce the growth of low concentrations of bacteria (i.e., 1×10^2 CFU), it may not have affected all bacterial cells at high concentrations (i.e., 5×10^4 CFU). These reasons should be clarified through further studies.

To determine whether the antibacterial activity of the fungal culture filtrate showed cytotoxic or cytostatic effects against *E. coli*, an MTT assay was performed. The results showed that the antibacterial compound did not completely suppress bacterial growth (Fig. 3). The data presented could not confirm the reason for the antibacterial activity of the fungal culture filtrate. Further studies need to be performed to elucidate this finding.

Antibacterial compound(s) produced from selected fungal isolates remained stable in response to heat and proteolytic enzyme treatments (Table 2). These results suggest that the antibacterial compound(s) is not a protein. Heat-stable antimicrobial compounds are not commonly produced. Some Class II bacteriocins have been reported to be heat stable (Bharti *et al*., 2012; Drider *et al*., 2006). Therefore, the antibacterial compound produced from our fungal isolates may have useful applications in various fields of study.

Fungi produce a wide range of compounds with biological activities against other organisms; most of these are products of secondary metabolites (Vey *et al*., 2001). In recent years, beauvericin and destruxins have become well-known secondary metabolites produced from many entomopathogenic fungi such as *B. bassiana* and *M. anisopliae*. These metabolites exhibit a wide variety of insecticidal, antibacterial, antifungal, anticancer, and antiviral activities (Pedras *et al*., 2002; Wang and Xu, 2012). These findings confirm the potential of entomopathogenic fungi as a source for lead structures of pharmaceutical interest. In our study, the antibacterial activity against *E. coli* was tested and demonstrated by using only entomopathogenic fungi that showed insecticidal activity against the great wax moth. Therefore, other biological activities should also be evaluated, and further investigation into the antibacterial activity compound(s) from our fungal isolates is also required. These studies will increase the usefulness of entomopathogenic fungi as biological control agents in addition to other biological applications.

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