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Screening and characterization of antimicrobial substances originated from entomopathogenic fungi

Tae Young Shin, Sung Min Bae, Soo Dong Woo *

Department of Agricultural Biology, College of Agriculture Life & Environment Sciences, Chungbuk National University, Cheongju 28644, Republic of Korea

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ABSTRACT

To illustrate the presence of different types of antimicrobial substances derived from entomopathogenic fungi, the antimicrobial activities of 342 fungi were evaluated, and their antimicrobial substances were analyzed. The antimicrobial activities of 342 fungi isolated under various conditions from different regions of Korea were evaluated against the bacterium *Bacillus cereus* and the fungus *Botrytis cinerea* using a dual culture technique on agar plates. The results showed that 170 isolates (49.7%) and 186 isolates (54.4%) inhibited the growth of *B. cereus* and *B. cinerea*, respectively. The culture filtrates of selected fungi completely suppressed the growth of the microorganisms, indicating the presence of antimicrobial substances. Characteristics of the antimicrobial substances such as thermostability, polarity, susceptibility to proteases and molecular weight varied not only within a culture filtrate but also with the fungal isolate. Different types of antimicrobial substances with different characteristics were present in the fungal culture filtrates. These substances may be useful not only as biocontrol agents against plant diseases but also in other industrial fields. This is the first study to demonstrate various kinds of antimicrobial substances from a large number of entomopathogenic fungi. This study suggests that novel antimicrobial substances could be isolated from entomopathogenic fungi.

Introduction

Entomopathogenic fungi are natural enemies of insects and contribute to the regulation of their host populations (Vega, 2008). These fungi are widely distributed such as aquatic, forest, and agricultural habitats, and are often used as active ingredients of microbial pesticide (Shah and Pell, 2003; de Faria and Wraight, 2007). Their mode of action against insects involves attachment of conidia to insect cuticle, followed by germination, cuticle penetration, and internal dissemination throughout the insect body (Vega et al., 2012). During this process, fungal secreted enzymes, protein toxins, and secondary metabolites can overcome the host immune system, modify host behavior, and defend host resources against competing pathogens and saprophytes (Isaka et al., 2005; Molnar et al., 2010).

Recently, several species of entomopathogenic fungi have been shown to have multiple roles in nature as endophytes, antagonists against plant pathogens and plant growth promoters (Vega et al., 2009; Ownley et al., 2010). In addition, the common entomopathogenic fungus *Metarhizium robertsii* could give a beneficial effect to plant by endophytic association with plant roots (Sasan and Bidochka, 2012). Potential antimicrobial effects of entomopathogenic fungi against microorganisms have also been recently reported (Lee et al., 2005; Goettel et al., 2008; Lozano-Tovar et al., 2013). Dual action against insect pests and plant pathogens were studied in *Beauveria* spp., *Metarhizium* spp., *Lecanicillium* spp. and *Clonostachys rosea* with good results (Kim et al., 2007, 2010; Lozano-Tovar et al., 2013; Keyser et al., 2015). Therefore, such fungi are not only entomopathogenic but also useful microorganisms in various fields and many researchers are suggesting new paradigms for the use of such organisms in various fields (Vega et al., 2009).

Although studies concerning bioactive substances from entomopathogenic fungi have been performed worldwide (Schmidt et al., 2003; Madla et al., 2005), they have been limited to a few fungal species and substances. In previous studies, we have isolated and identified 342 entomopathogenic fungal isolates including 28 species belonging to 20 genera from various habitats in Korean soils, and some of them isolates showed antibacterial, antioxidant and anticancer activities (Shin et al., 2013a,b, 2014). In this study, we tried to suggest the presence of different types of the antimicrobial substances from these entomopathogenic fungi using the bacterium *Bacillus cereus* and the fungus *Botrytis cinerea*. Both microorganisms are used for the antimicrobial screening test in many studies (Wilson et al., 1997; Lee et al., 2005). Especially, *B. cinerea* is a serious fungal pathogen causing gray mold diseases in many plant species and is difficult to control because of its various modes of attack (Dean et al., 2012). This study will serve as a guide for future entomopathogenic fungal resource programs.

* Corresponding author.
E-mail address: sdwoo@cbnu.ac.kr (S.D. Woo).

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Materials and methods

Microorganisms

The bacterium *Bacillus cereus* used for the antibacterial assays in this study was isolated from soil and cultured using BG media (1 g casamino acids, 10 g peptone, and 5 g dextrose in 1000 ml distilled water, pH 7.4). One ml of an overnight bacterial culture was inoculated into 100 ml of media and grown to mid-exponential phase ($O.D._{650\ nm} = \sim 0.5$) at 25 °C with shaking at 150 rpm.

The fungal isolate *Botrytis cinerea* GBYP-8 was obtained from the plant fungal disease laboratory of Chungbuk National University, Korea and used for the antifungal assay. To investigate their antimicrobial activities, 342 entomopathogenic fungal isolates from 20 different genera were used (Shin et al., 2013b). The entomopathogenic fungi were maintained on Potato dextrose agar (PDA) media at 25 °C in the dark.

Fungal culture filtrates

Fungal conidia were obtained by scraping a 2 week-cultured PDA plate and suspending the material in a 0.05% Tween-80 solution. The conidial suspension was vigorously agitated and filtered through cotton to remove the mycelial debris. After counting the number of conidia, 50 ul of the conidial suspension (2×10^6 conidia/ml) inoculated in 20 ml Sabouroud dextrose yeast broth (SDYB) (10 g Bacto-peptone, 40 g dextrose, and 10 g yeast extract in 1000 ml distilled water, pH 6.0) in a 100 ml flask. The samples were cultured at 25 °C in the dark and shaken at 150 rpm for 10 days. The percentage of viable conidia was determined prior to inoculation by conidia germination assay; in all cases, more than 90% of the conidia were determined to be viable. After 10 days, the samples were centrifuged at 10,000 ×g for 20 min at 4 °C, and the supernatants were filtered using a 5 um membrane filter (Advantec No. 2, Japan) to separate the crude extract from the mycelial and spore mass. Subsequently, the culture filtrates were adjusted to pH 5.1 and pH 7.4 with HCl and NaOH for the antibacterial and antifungal assays, respectively, and re-filtered through a 0.45 um membrane filter (Advantec Dismic-25cs, Japan). All cell-free fungal culture filtrates were stored at −70 °C until used.

Antibacterial activity assay

To detect antibacterial activity, two Ø6 mm agar plugs containing fungi from 5 day-old PDA were placed 3 cm away from the center of a 90 mm petri dish containing SDYA and incubated at 25 °C in the dark for 2 days. After incubation, a *B. cereus* bacterial suspension (1×10^8 CFU/ml) was inoculated in the center of each dish using a spreader. The plate was incubated at 25 °C in the dark for 24 h, and the clear inhibition zone was measured.

For the antibacterial assay of fungal culture filtrates, the *B. cereus* was diluted with medium to 5×10^5 CFU/ml. One hundred ul of bacterial suspensions was plated on each well of 96-well plates with 100 ul of fungal culture filtrate at different concentrations (1%, 10%, 20%, 40%, 60%, 80%, and 100%) diluted with SDYB and incubated at 25 °C for 16 h. The absorbance of each well was measured at 650 nm using a microplate reader (Molecular Devices, UK). For the control, bacterial suspensions were cultured in SDYB with different amounts of Dulbecco's PBS in place of the fungal culture filtrate.

Antifungal activity assay

A dual culture method was used to detect the antifungal activity against *B. cinerea* on SDYA. A Ø6 mm agar plug containing 1 week-old *B. cinerea* was placed at the center of an SDYA plate and two agar plugs containing different test fungi were placed 3 cm away from center plug that contained *B. cinerea*. The plate was incubated at 22 °C in the dark for 4 days, and the clear inhibition zone was measured.

For the antifungal assay of the fungal culture filtrates, conidial suspensions of *B. cinerea* were diluted with Potato dextrose broth (PDB, pH 5.1) to 2×10^4 conidia/ml. One hundred ul of the conidial suspension was plated on each well of 96-well plates with 100 ul of fungal culture filtrate at different concentrations (1%, 10%, 20%, 40%, 60%, 80%, and 100%) and incubated at 22 °C for 48 h. The absorbance of each well was measured at 595 nm using a microplate reader. For the control, fungal conidial suspensions were cultured in SDYB medium with different concentrations of 20 mM citrate phosphate buffer in place of the fungal culture filtrate.

Characterization of the antimicrobial substances

To determine its heat stability, the culture filtrate was subjected to various temperatures (50 °C, 80 °C, 100 °C, and 121 °C) for 15 min. After the heat shock, the culture filtrate was quickly cooled to 25 °C, and its antimicrobial activity was evaluated.

The hydrophilic/hydrophobic characteristics of the antimicrobial substances were determined by an acetone precipitation method. Four volumes of cold (−20 °C) acetone (Sigma, USA) was added to one volume of sample. The mixture was vortexed and incubated at −20 °C for 1 h. After incubation, the sample was centrifuged at 13,000 ×g for 10 min at room temperature, and the pellet and supernatant were separated. The pellet was air dried for 30 min and the supernatant was evaporated by nitrogen. Both samples were adjusted to the original volume with distilled water, and then used in the antimicrobial activity assays.

To test their protease stability, the culture filtrate was treated with proteinase K (Sigma, USA) or pronase E (Sigma, USA) at a final concentration of 1 mg/ml and 300 μg/ml at 37 °C for 2 h and 24 h, respectively. Then, the sample was treated at 121 °C for 15 min or 80 °C for 20 min to inactivate proteinase K and pronase E, respectively, before the antimicrobial activity assay was carried out.

The molecular weight of an antimicrobial substance was estimated using an Amicon ultra centrifugal filter device (Molecular wt. cut off: 30 kDa, 3 kDa) (Millipore Corporation, Ireland). After ultrafiltration, the samples were adjusted to the original volume with distilled water, and then used in the antimicrobial activity assay.

Results

Screening of fungi for antimicrobial activity

The antimicrobial activity of 342 entomopathogenic fungal isolates against *B. cereus* and *B. cinerea* was investigated on agar plates. The results indicated that the antimicrobial activities varied (Figs. 1, 2). Of the 342 isolates tested, antibacterial activity was presented in 49.7% (170) of the isolates. The antibacterial activity was divided into three categories; high activity with a clear zone larger than 10 mm (16 isolates, 4.7% frequency), medium activity with a clear zone of 5 to 9.9 mm (108 isolates, 31.6% frequency) and low activity with a clear zone less than 5 mm (46 isolates, 13.5% frequency). Antifungal activity to *B. cinerea* was present in 54.4% (186 isolates) of the isolates. The antifungal activity was also divided into three groups; high-activity with a clear zone greater than 5 mm (14 isolates, 4.1% frequency), medium activity with a clear zone of 2 to 4.9 mm (90 isolates, 26.3% frequency) and low activity with a clear zone less than 2 mm (82 isolates, 24% frequency). The antimicrobial activities observed in the various fungal isolates varied with genus and species (Tables 1, 2). The degree of antibacterial and antifungal activity in the various fungal isolates was different from each other. Antibacterial activity was more common in *B. brongniartii*, *I. farinose*, *I. fumosorosea* and *M. anisopliae* (Table 1), but antifungal activity was more common in *B. bassiana* and *Isaria* spp. (Table 2). The fungal isolates which showed the antifungal activity (186 isolates, 54.4% of