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Culture Filtrates of *Trichoderma* Isolate H921 Inhibit *Magnaporthe oryzae* Spore Germination and Blast Lesion Formation in Rice

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Abstract

Spore germination and appressorium formation of *Magnaporthe oryzae* spores was completely suppressed by an ethyl acetate extract of the culture filtrate from the H921 isolate (H921-EAE-CF). Production of antifungal substance(s) in the H921-EAE-CF began to increase up to 3 days after isolate H921 incubation. Furthermore, heat treatment (105˚C or 121˚C) of H921-EAE-CF did not alter its inhibitory effect on *M. oryzae* spore germination compared to non-heat-treated H921-EAE-CF. Blast lesion formation inhibition by H921-EAE-CF was dose-dependent. Internal transcribed spacer (ITS) region sequence analysis indicated that this isolate shared similarities with species of the genera *Trichoderma.* This study suggests that H921-EAE-CF contains some antifungal substances that could be promising candidates for control of rice blast disease.

Keywords

Culture Filtrate, *Magnaporthe oryzae*, *Trichoderma* sp., Rice

1. Introduction

The plant pathogenic fungus *Magnaporthe oryzae* infects rice and is considered one of the most devastating causes of disease in this crop species [1]. The application of fungicides and the use of resistant rice cultivars are generally effective control methods. However, the durability of genetic resistance in improved rice cultivars is often short lived in the field because of the pathogen's ability to rapidly evolve to overcome resistance. On the other hand, the

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use of synthetic fungicides to control plant pathogens has induced fungal resistance [2] [3]. Therefore, a search for antifungal substances is required to develop new fungicides. Antifungal substances of microbial origin can play an important role in the biological and chemical control of plant diseases [4] [5]. In addition, bacterial and fungal antagonists have been used to control fungal diseases on various crops. For example, culture broth and culture filtrates of *Streptomyces philanthi* RM-1-138 inhibited sheath blight disease in rice caused by *R. solani* Kühn [6]. Likewise, *Staphylococcus* sp. strain LZ16, *Bacillus subtilis* IK1080 strain, *Streptomyces* spp., *Sphingomonas* sp., *Chryseobacterium* sp., and *Trichoderma* spp. have all been used as biocontrol agents for rice blast [7]-[11].

In our previous paper, we reported that ethyl acetate extract of culture filtrate of symbiotic and parasitic fungi from wild mushrooms may yield potential control agents for plant diseases such as the rice blast disease [12]. In addition, we recently isolated the *Trichoderma* isolate H921 (closely related to the type strain of *Trichoderma koningiopsis*) from wild mushrooms. It is well known that *Trichoderma* spp. produce high molecular weight enzymes such as chitinase and *β*-1,3-glucanase that mediate cell wall and cell membrane damage in plant pathogens. Also, *Trichoderma* spp. produce low molecular weight antifungal compound such as 6-pentyl-*α*-pyrone and viridiofungin A. However, antifungal activity of the low molecular weight compounds against rice blast fungus produced by *T. koningiopsis* has not yet been clearly elucidated.

In this paper, we report that ethyl acetate extract of culture filtrate of the *Trichoderma* isolate H921 isolated from wild mushrooms can protect rice from rice blast fungus, *M. oryzae*.

2. Material and Methods

2.1. Microorganisms and Preparation of the Culture Filtrate

Isolate H921 was isolated from *Amanita pantherina* var. *pantherina* of wild mushroom samples collected from a paddy field by the method described previously [12]. Single spore was collected from isolate H921 and maintained on potato sucrose agar (PSA) slants until use. Mycelial discs of isolate H921 were grown on PSA medium (200 g potato, 20 g sucrose, 20 g agar, 1 L distilled water) and used to individually inoculated 30 mm test tubes containing 20 mL potato sucrose broth (PSB). The liquid cultures were incubated at 27˚C in the dark for 1 - 7 days with constant shaking on a rotary shaker (130 rpm). A crude culture filtrate (20 mL) was extracted twice with 20 mL of ethyl acetate. The ethyl acetate fraction was added to distilled water and evaporated at 45˚C under reduced pressure until only the water fraction remained. The aqueous volume was adjusted to 4 (5-fold), 10 (2-fold), 20 (1-fold) or 40 (1/2-fold) mL. Adjusted aqueous samples were used as an ethyl acetate extract of the culture filtrate from the isolate H921 (H921-EAE-CF) in this experiment. As a control, ethyl acetate extracts of PSB (un-inoculated medium) were prepared by method a described above.

2.2. Antifungal Activity of the Culture Filtrate

M. oryzae (strain Naga 69 - 150, race 007) was grown on rice bran agar at 27˚C for 14 days, washed with running water to remove aerial hyphae, and kept at 27˚C under near-ultraviolet radiation (FL20s BL-B; Panasonic, Osaka, Japan) for 2 days to induce sporulation.

M. oryzae spores (1×10^5 spores/mL) suspended in the H921-EAE-CF (2-fold) or ethyl acetate extract of PSB (2-fold) were dropped onto glass slides and kept in a moist chamber at 27˚C. After 24 h incubation, the percentage of spore germination and appressorium formation were determined by light microscopy [7].

2.3. Heat Treatment

To investigate the effect of heat treated H921-EAE-CF on the spore germination of *M. oryzae*, a 2-fold concentration of H921-EAE-CF was heated at 105˚C or 121˚C for 20 min using an autoclave. Antifungal activity was measured using the method described above. Non-heat treated and heat-treated 2-fold concentrations of ethyl acetate extracts of PSB (un-inoculated medium) were prepared by the method as described above, and it was used as controls.

2.4. Co-Treatment with Culture Filtrate on *M. oryzae* to Induce Lesion Formation in Rice Leaves

Isolate H921 was cultured for 7 days and H921-EAE-CF was prepared. The spores of *M. oryzae* were prepared

as described above. *M. oryzae* spores (1×10^5 spores/mL) were suspended with 1/2, 1, 2, or 5-fold concentrations of H921-EAE-CF. A 5-fold concentration of ethyl acetate extract of PSB was used as a control. The third leaf of rice plants (*Oryza sativa* "Koshihikari") was prepared as described previously [13] [14]. Rice plants were inoculated with the *M. oryzae*/H921-EAE-CF mixtures and incubated in a moist chamber for 24 h at 26˚C in the dark. Disease severity was observed 5 days after inoculation. Disease severity index [15] of blast diseases was assessed according to the formula $\{(4A + 3B + 2C + D)/(4 \times 15)\} \times 100$, where A = lesion area of >50%, B = lesion area of 20% - 50%, C = lesion area of 10% - 20%, D = lesion area of 0.5% - 10%, and E = no lesions.

2.5. DNA Extraction, PCR Amplification, Sequencing and Creating Phylogenetic Tree

To identify the active isolate H921, the sequence of the internal transcribed spacer (ITS) region (including 5.8S rDNA) was determined by PCR with the ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers. Fungal genomic DNA was extracted from the mycelia as described by Suzuki *et al*. [16], and used as the PCR template. PCR amplification of the ITS region was performed as follows: an initial step of 5 min at 95˚C; followed by 30 cycles of denaturation at 94˚C, for 1 min, annealing at 62˚C, for 1 min, and elongation at 72˚C, for 1 min; and a final step for 5 min at 72˚C. PCR-amplified fragments were purified by using the HiYield Gel/PCR DNA fragment extraction kit (RBC Bioscience, Taipei, Taiwan). DNA sequencing was performed with a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). DNA sequence analysis was performed on an ABI PRIZM 3130*xl* Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). Sequence homology was determined by searching with the BLAST suite of programs (DNA Data Bank, Japan). Phylogenetic tree was constructed using the neighbor-joining method [17].

2.6. Statistical Analysis

Data are reported as mean ± standard deviation (SD). Significant differences from the experimental values were determined by a Tukey-Kramer test using SPSS Statistics ver. 19.0 for Windows (IBM, Armonk, NY, USA).

3. Results

3.1. Effect of H921-EAE-CF on *M. oryzae* Infection Behaviors

H921-EAE-CF had effect spore germination and appressorium formation of *M. oryzae* (**Figure 1**). Furthermore, only at concentrations of the original volume (1-fold) and over did H921-EAE-CF inhibited spore germination and appressorium formation (data not shown). In addition, we examined the effect of heat treatment had on the ability of H921-EAE-CF (2-fold) to suppress spore germination and appressorium formation. In the non-heat treated control (unheated) and PSB heat-treated (105˚C and 121˚C) controls, spore germination rates were 94.7% ± 6.2%, 98.1% ± 2.3%, and 98.2% ± 2.0%, respectively (**Figure 1(a)**). In contrast, the rates of spore germination in non-heat treated (unheated) and heat-treated H921-EAE-CF were 6.0% ± 4.7%, 4.9% ± 3.9% (after 105˚C), and 4.2% ± 2.9% (after 121˚C), respectively (**Figure 1(a)**). The rates of appressorium formation in the three control groups (unheated, 105˚C and 121˚C) were 79.6% ± 25.1%, 91.1% ± 15.6%, and 97.8% ± 2.6%, respectively (**Figure 1(b)**). The appressorium formation rates of the samples in the three treatment groups were 0.3% ± 0.8%, 0.1% ± 0.5%, and 0.1% ± 0.5%, respectively (**Figure 1(b)**). Thus, the heat treatment of the H921-EAE-CF did not reduce its inhibitory effect against *M. oryzae*.

3.2. Effect of Culture Age on the Antifungal Activity of the H921-EAE-CF

We next investigated the effect of longer culture periods on the activity of the H921-EAE-CF. In the PSB control, the rates of spore germination and appressorium formation were 99.4% ± 1.1% and 98.0% ± 2.8%, respectively (**Figure 2(a)**, **Figure 2(b)**). The rates of spore germination after treatment with H921-EAE-CF of shaken culture after 1 day incubation period were 99.3% ± 1.3% (**Figure 2(a)**). The rates with the 3, 5, or 7 day H921-EAE-CF were 1.7% ± 2.7%, 6.5% ± 4.8%, and 6.0% ± 0.3% respectively (**Figure 2(a)**). Appressorium formation occurred at a rate of 98.9% ± 1.7% for the 1 day H921-EAE-CF (**Figure 2(b)**). The rates of appressorium formation for the 3, 5, and 7 days of H921-EAE-CF were 0.5% ± 1.5%, 4.8% ± 9.4%, and 0.3% ± 0.8%, respectively (**Figure 2(b)**). The analysis indicates that presence of antifungal substances in the H921-EAE-CF increased with the time in culture of isolate H921 with an optimum time of 3 days.

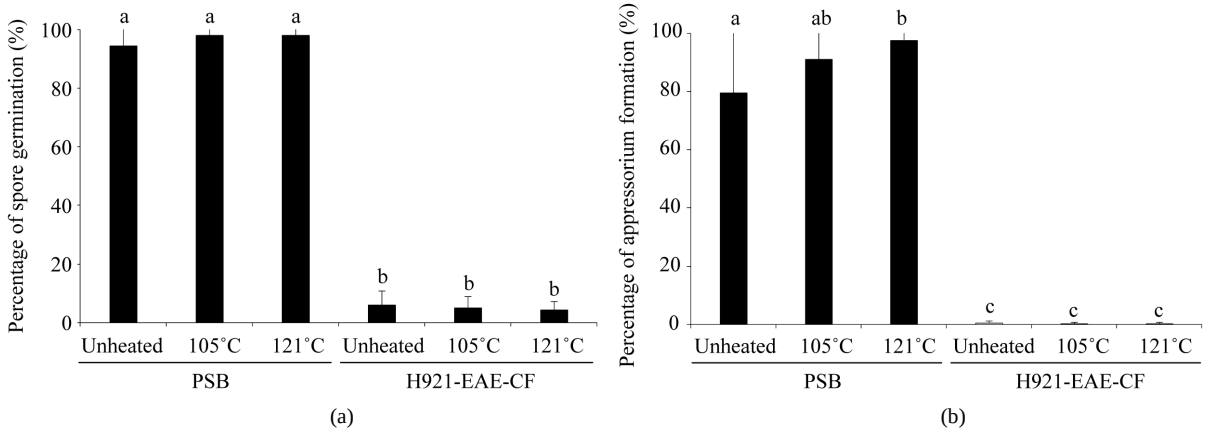

Figure 1. The ethyl acetate extract of *Trichoderma* isolate H921 culture filtrates inhibit the infective behavior of *Magnaporthe oryzae*. Spores of *M. oryzae* were suspended in the different culture filtrates (EAE-CF) and dropped on glass slides. After 24 h of incubation in a moist chamber, the spore germination (a) and appressorium formation (b) were observed by light microscopy. The rates of spore germination and appressorium formation per germinating spore were calculated. Experiments were repeated three times and a total of 300 spores per experiment were examined. The bar at the top of each column represents the standard deviation of the mean. Means followed by different letters are significantly different using the Tukey-Kramer test ($p < 0.05$).

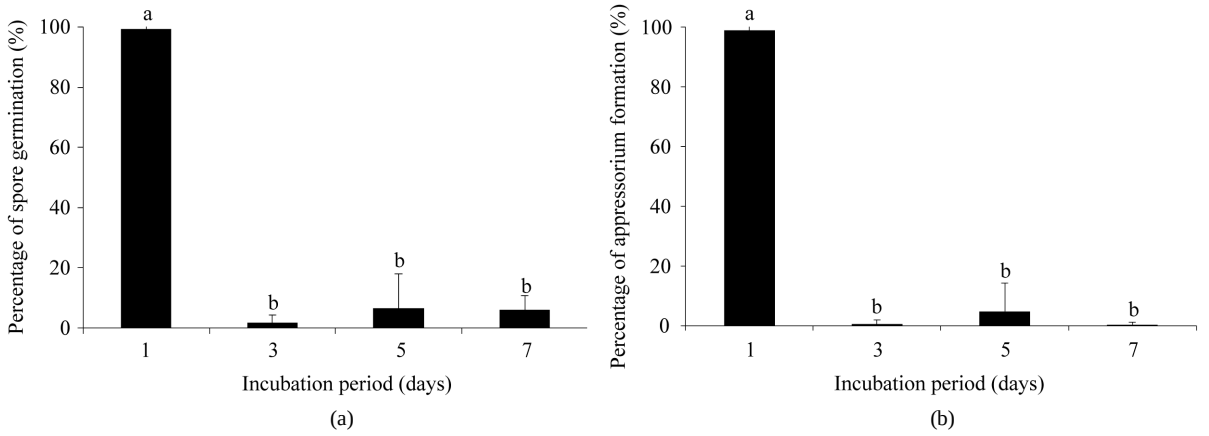

Figure 2. Time course of inhibition activity of the ethyl acetate extract of culture filtrates (EAE-CF) of the H921 isolate on *Magnaporthe oryzae* infection. Spores of *M. oryzae* were suspended in EAE-CF for different lengths of time and dropped on glass slides. After 24 h of incubation in a moist chamber, the spore germination (a) and appressorium formation (b) were observed by light microscopy. The rates of spore germination and appressorium formation per germinating spore were calculated. Experiments were repeated three times and a total of 300 spores per experiment were examined. The bar at the top of each column represents the standard deviation of the mean. Means followed by different letters are significantly different using the Tukey-Kramer test ($p < 0.05$).

3.3. Effect of Co-Treatment with H921-EAE-CF on Lesion Formation by *M. oryzae* on Rice Leaves

Rice leaves (cultivar "Koshihikari") were inoculated with *M. oryzae* spores in the presence of different concentrations of H921-EAE-CF (1/2-fold, 1-fold, 2-fold, 5-fold). In controls, rice leaves sprayed with a 5-fold concentration of PSB showed a disease severity of 99.5% ± 0.8% (**Figure 3**). Disease severity in these treatments groups, 1/2-fold, 1-fold, 2-fold and 5-fold were 31.9% ± 18.9%, 16.7% ± 12.7%, 7.6% ± 12.6%, and 3.3% ± 7.4%, respectively (**Figure 3**). Thus, H921-EAE-CF inhibited blast lesion formation in dose-dependent manner.

4. Discussion

Trichoderma species have been reported as biocontrol agents for numerous plant pathogens and this genus is often

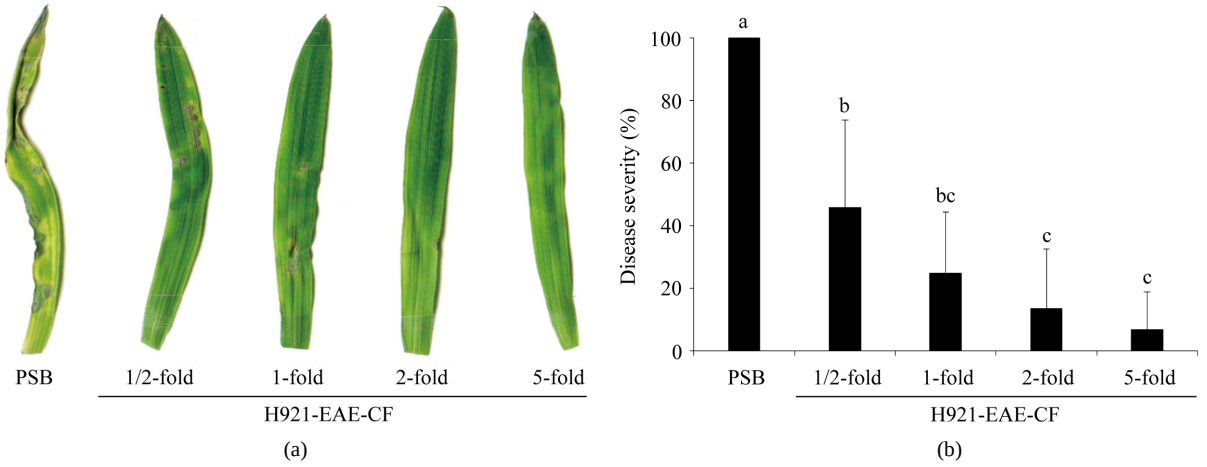

Figure 3. Inhibition of *Magnaporthe oryzae* lesion formation on rice leaves by the ethyl acetate extract of the culture filtrates (EAE-CF) of H921 isolate. Rice plants were inoculated with *M. oryzae* in the presence of H921-EAE-CF isolate and kept in a moist chamber for 24 h. After 5 days, lesion formation (a) and disease severity (b) was measured. Data are representative of the mean values of the results from three separate experiments with 15 rice plants in each treatment per experiment. The bar at the top of each column represents the standard deviation of the mean. Mean values followed by different letters are significantly different using Tukey-Kramer test ($p < 0.05$).

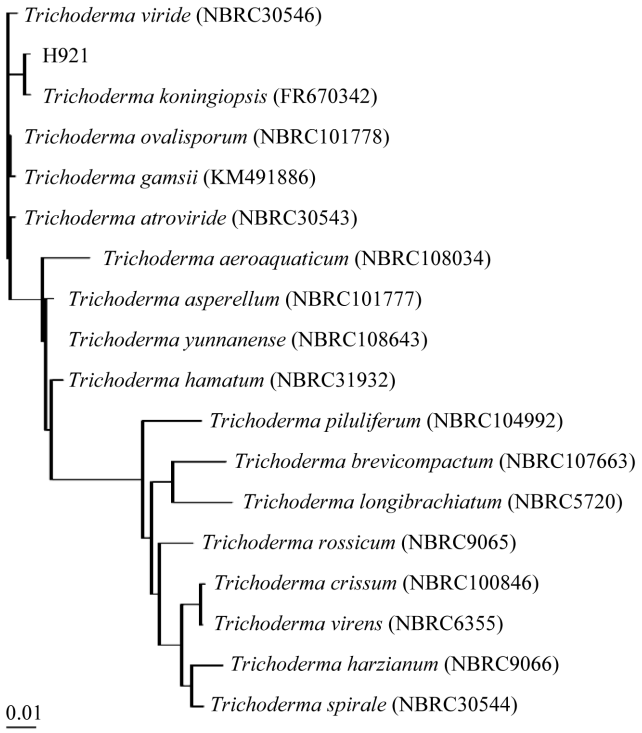

Figure 4. Phylogenetic tree based on ITS sequences of H921 isolate. A bootstrap consensus Neighbor-Joining tree for the *Trichoderma* isolate H921 was created based on the Kimura 2-Parameter distance matrix (1000 replicates). The *Trichoderma viride* (NBRC30546) was used as the out-group. The scale bar represents 1% sequence dissimilarity.

employed as a biological control in agriculture, as an alternative to synthetic chemical products. In general, these isolates characteristically secrete hydrolytic enzymes such as chitinases, glucanases, and protease that are capable of hydrolyzing the cell walls of phytopathogenic fungi [18]-[21].

However, chemical stability of antifungal substance in natural environmental conditions is important for develop new fungicide for plant disease.

When the effect of ethyl acetate extract of the culture filtrate from the H921 isolate (H921-EAE-CF) on infection behaviors of *M. oryzae* to use a glass slide were investigated, both spore germination and appressorium formation of *M. oryzae* were inhibited by H921-EAE-CF. In addition, antifungal substance(s) of H921-EAE-CF was heat-stable substance(s). In our present study, when H921-EAE-CF was separated by organic solvent system on a thin layer chromatography plate, more than two antifungal substances to *M. oryzae* were observed (data not shown). This result suggested that one or more antifungal substances can be more easily from the isolated microorganisms using potato sucrose broth (PSB) medium. To identify isolate H921, we used specific primers to PCR amplify the ITS region including 5.8S rDNA by using its genomic DNA. Phylogenetic analyses showed that the microorganism was most closely related to the type strain of *T. koningiopsis* (**Figure 4**) and therefore, the isolate H921 was identified as a member of the genus *Trichoderma*. The role of metabolites of *T. koningiopsis* in antifungal activity against rice blast fungus has not yet been clearly elucidated. As mentioned above, antifungal substance(s) included in H921-EAE-CF are heat-stable. Interestingly, some *Trichoderma* spp. have been reported to produce 6-pentyl-*α*-pyrone, which inhibits the growth of plant pathogens such as *R. solani*, *Fusarium oxysporum*, *Botrytis* sp., and *Athelia rolfsii*. Another compound, viridiofungin A, has been shown to be the active compound against *F. moniliforme* in the T23 isolate of *T. harzianum* [22]. Analysis of H921-EAE-CF by thin layer chromatography indicated that the antifungal substance included in H921-EAE-CF differs from that of 6-pentyl-*α*-pyrone (data not shown). In addition, there are no reports on the utility of viridiofungin A for control of rice blast fungus, and blast lesion formation was significantly inhibited in the presence of H921-EAE-CF. Also, in this study, the H921-EAE-CF showed no phytotoxic activity in rice. These result suggested that antifungal substance(s) included in H921-EAE-CF may contribute to develop new fungicide for rice blast.

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