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Suppression effects of a secondary metabolite of *Biscogniauxia* sp. strain O-811 obtained from mushrooms against the rice blast fungus *Magnaporthe oryzae*

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Abstract Rice blast disease, caused by *Magnaporthe oryzae*, is considered the most important fungal disease of rice worldwide. Owing to continuous resistance acquisition, several fungicide chemicals are no longer effective. Therefore, there is a need to identify natural components for the development of new agents for the control of fungal pathogens. In the present study, we evaluated the antifungal activity of an ethyl acetate extract of the culture filtrates (ECF) of strain O-811 isolated from the fresh fruiting bodies of wild mushrooms against the rice pathogen *M. oryzae*. When conidia of *M. oryzae* were suspended in O-811-ECF and dropped onto glass slides, conidia germination was significantly inhibited in a dose-dependent manner. Additionally, the length of blast lesions produced by *M. oryzae* was significantly reduced in the presence of O-811-ECF compared to distilled water. Internal transcribed spacer region sequence analysis indicated that this isolate (O-811) shared similarities with the fungal species of *Biscogniauxia* sp. The growth inhibition zone against *M. oryzae* in the presence of O-811-ECF was observed at Rf 0.66 on the thin layer chromatography (TLC) plate. Furthermore, the growth inhibition zone by O-811-ECF and{(3aS,4aR,8aS,9aR)-3a-hydroxy-8a-methyl-3,5-dimethylenedecahydronaphto[2,3-b]furan-2(3H)-one} (HDFO) was detected with the same Rf value on TLC plates. These results suggest that the inhibitory compound included in the strain of the O-811 isolate of *Biscogniauxia* sp. is the same as that from the HDFO of strain O-821.

Keywords: *Biscogniauxia* sp., Plant disease control, Rice blast fungus, Secondary metabolite

Introduction

Rice blast disease, caused by *Magnaporthe oryzae*, is considered the most important fungal disease of rice (Ou, 1985). *Magnaporthe oryzae* has been responsible for the outbreak of rice blast in 85 countries, causing 10–35 % loss in harvests (Fisher et al., 2012). Besides rice, fungal outbreaks have occurred in wheat, barley, and millet (Couch et al., 2005). The key to the chemical control of rice blast disease is to overcome fungicide-resistant pathogens (So et al., 2002). Therefore, there is a need for antifungal compounds that can be developed into new fungicides. Antifungal compounds of microbial origin play an important role in the biological and chemical control of plant diseases (Fravel, 1998; Shimizu et al., 2000; Uddin and Viji, 2002; Chaijuckam and Davis, 2010).

Recently, we reported the existence of symbiotic and parasitic fungi in wild mushrooms, and their potential for the control of plant diseases.

The objective of this study was to investigate the suppression effects of a culture filtrate of strain O-811 obtained from mushrooms against the rice blast fungus *M. oryzae*.

Materials and Methods

Microorganisms and plants

The strain O-811 was obtained from the fresh fruiting bodies of wild mushrooms collected from a paddy field by the method previously described by Nguyen et al. (2017). A single colony of strain O-811 was collected and maintained on potato sucrose agar (PSA) slants until its use.

A virulent strain of *Magnaporthe oryzae* (strain Naga 69-150, race 007) was grown on rice bran agar at 26 ± 2 ℃ for 10 days, washed with running distilled water to remove aerial hyphae, and maintained at 26 ± 2 ℃ under near-ultraviolet radiation (FL20s BL-B; Panasonic, Osaka, Japan) for 2–4 days to induce sporulation.

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Seedlings of *Oryza sativa* L. 'Koshihikari' were used in this study. Seedlings were grown in a glasshouse for two weeks as previously described by Fujita et al. (1994).

Culture filtrate of isolated microorganism

The mycelial disks (8 mm diameter) of strain O-811 were grown on a PSA medium [extract from 200 g/L potato, 2 % (w/v) sucrose, 2.0 % (w/v) agar], and they were individually inoculated in test tubes containing 500 mL of potato sucrose broth (PSB). The liquid cultures were incubated at 26 ± 2 ℃ in the dark with constant shaking on a rotary shaker (130 rpm). A crude culture filtrate (500 mL) was extracted twice with 500 mL of ethyl acetate. The ethyl acetate fraction was added to distilled water and subjected to evaporation at 45 ℃ under reduced pressure until only the water fraction remained. The aqueous volume was adjusted to 5 (100-fold), 25 (50-fold), 50 (10-fold), 100 (5-fold), or 500 (1-fold) ml. The adjusted aqueous samples were used as an ethyl acetate extract of the culture filtrate from strain O-811 (O811-ECF).

Influence of the O-811 ECF on the spore germination of *M. oryzae*

Magnaporthe oryzae conidia (1×10^5 conidia/mL) suspended in different concentrations of the O-811-ECF (1, 5, 10, 50, and 100-fold) were dropped onto glass slides and maintained in a moist chamber at 26 ± 2 ℃. After 24 h, the percentage of conidial germination was determined under a light microscope and calculated using the following formula: (numbers of conidia germinated / total number of conidia) × 100. The experiments were repeated three times.

Suppression effect of the O-811-ECF on the blast lesion development in rice leaves

Seedlings of *O. sativa* L. 'Koshihikari' were grown as described above. *Magnaporthe oryzae* conidia (1×10^5 conidia/mL) suspended in the absence (control) or presence of the O-811-ECF were dropped (10 µL/drop) onto the rice leaves. The inoculated rice plants were incubated in a moist chamber for 24 h at 26 ± 2 ℃ in the dark and then maintained under 11 h light and 13 h dark conditions. The diameter of blast lesions on rice leaves was measured 7 days after inoculation. The experiments were independently repeated three times. A total of ten rice blast lesions per experiment were examined.

DNA extraction, PCR amplification, sequencing, and creation of phylogenetic tree

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. (2006) and used as the PCR template. The PCR amplification of the ITS region was performed as follows: an initial step of 5 min at 95 ℃; followed by 30 cycles of denaturation at 94 ℃ for 1 min, annealing at 62 ℃ for 1 min, and elongation at 72 ℃ for 1 min; and a final step of 5 min at 72 ℃. The PCR-amplified fragments were purified using the NucleoSpin® Gel and PCR Clean-up Kit (Takara-Bio, Shiga, Japan). The DNA sequencing was performed with a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). The DNA sequence analysis was performed on an ABI PRIZM 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). Sequence homology was determined by searching with the BLAST suite of programs (DNA Data Bank, Japan). The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987).

Detection of inhibitory compounds with Thin Layer Chromatography (TLC)

Silica gel TLC plates (Silica gel 60, Merck KGaA, Darmstadt, Germany) were used for the detection of potential antifungal compounds (Nguyen et al., 2017). Two hundred microliters (100-fold) of the O-811-ECF were spotted onto TLC plates and then developed using an ethyl acetate and toluene (1:1, v/v) solvent system. After development, inhibitory compounds were detected on the TLC plates by a growth inhibition assay of *M. oryzae* conidia. In brief, the TLC plates were sprayed with a concentrated conidia suspension of *M. oryzae* in the presence of PSA containing the antibiotic chloramphenicol to avoid bacterial contamination. The inoculated plates were kept in a moist chamber at 26 ± 2 ℃. After 7 days of incubation, the growth-inhibition zones were observed on the white TLC plate background.

Statistical analysis

Data are reported as mean ± standard deviation (SD). Significant differences were determined by t-test ($P < 0.05$) or Tukey-Kramer test ($P < 0.05$) using the statistical package for the social sciences (IBM SPSS version 22.0).

Results and Discussion

To determine the inhibitory activity of strain O-811-ECF, the conidia germination of *M. oryzae* spores in the presence of the O-811-ECF was investigated. The percentage of *M. oryzae* conidia germination in 1-, 5-, 10-, 50-, and 100-fold concentrated O-811-ECF were 97.6 ± 1.8, 95.9 ± 3.3, 86.1 ± 5.2, 17.4 ± 9.1, and 6.5 ± 2.4 %, respectively (Fig. 1). In contrast, in the control, the percentage of spore germination in distilled water was 93.2 ± 5.1 % (Fig. 1). We also determined the inhibitory activity of heat-pretreated (121 ℃, 20 min) O-811-ECF on conidia germination to determine whether the active compounds are heat stable (data not shown). The results indicated that the strain O-811 produced heat-stable inhibitory compounds against *M. oryzae*.

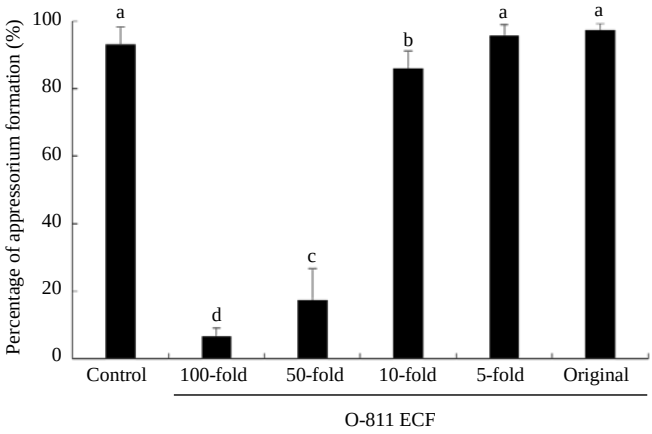

Fig. 1 Inhibitory activity of the ethyl acetate extracts of culture filtrate of the strain O-811 (O-811 ECF) on the infection behaviors of *Magnaporthe oryzae*. Conida of *M. oryzae* were suspended in different concentration of O-811 ECF and dropped on glass slides. After 24 h of incubation in a moist chamber, Conidia germination was observed by light microscopy. Experiments were repeated at least three times. Bars represent ±SD. Means followed by different letters are significantly different according to the (Scheffe's test, P<0.05).

To elucidate the suppression effect of the O-811-ECF to *M. oryzae* in rice, rice leaves were inoculated with *M. oryzae* conidia in the presence of O-811-ECF. Compared to the control, the presence of O-811-ECF (100-fold) significantly decreased the blast lesion formation (Fig. 2). Blast lesion length in the presence of O-811-ECF was 0.7 ± 1.3 mm (Fig. 2). In contrast, rice leaves inoculated with *M. oryzae* showed a blast lesion length of 3.4 ± 3.0 mm. Additionally, O-811-ECF did not show a phytotoxic effect on rice at the used concentration (data not shown). This result suggests that the inhibitory compound from O811-ECF inhibits blast lesion formation on rice leaves.

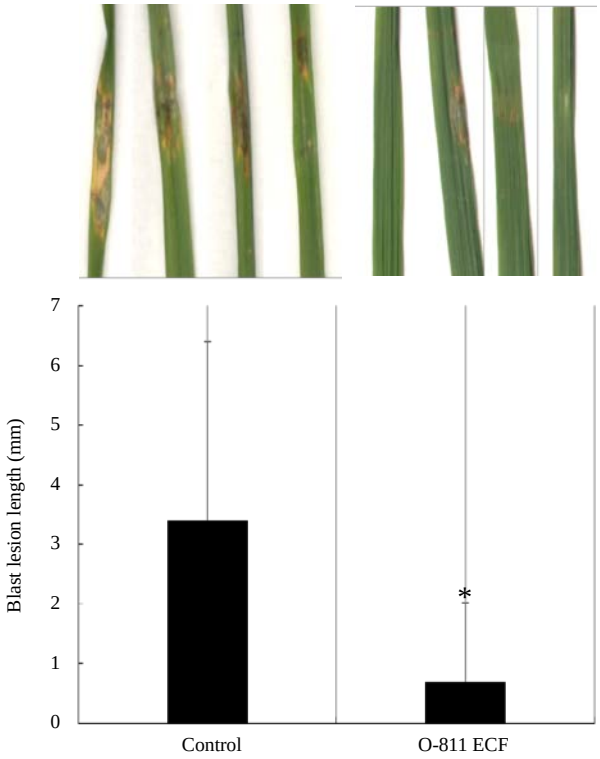

Fig. 2 Suppressive effect of the ethyl acetate extracts of culture filtrate of the strain O-811 (O-811 ECF) on lesion formation of *Magnaporthe oryzae* in rice leaves. Rice leaves were inoculated with *M. oryzae* in the presence of O-811 ECF (100-fold) or distilled water. After 7 days, blast lesion lenght were investigated. The data are the means of the results of at least three experiments. Bars represent ±SD. Asterisk indicate significant difference compared to control (t-test, $P < 0.05$).

To identify strain O-811, we used specific primers to amplify the ITS region, including 5.8S rDNA, by using its genomic DNA. Phylogenetic analyses showed that the microorganism was most closely related to *Biscogniauxia* sp. HHP54 (JN687972) (Fig. 3). Therefore, the strain O-811 was identified as a member of the genus *Biscogniauxia*. Recently, we found that the ECF of strain O-821 (*Biscogniauxia* sp.) isolated from mushrooms inhibited the infection behavior of *M. oryzae* and blast lesion formation in rice, and identified

{(3aS,4aR,8aS,9aR)-3a-hydroxy-8a-methyl-3,5-dimethylene decahydronaphto[2,3-b]furan-2(3H)-one} (HDFO) as an inhibitory compound with suppression effects against *M. oryzae* in rice (Nguyen et al., 2018). However, the production of HDFO in ECF of strain O-811 has not yet been elucidated. Therefore, we investigated whether strain O-811 produces HDFO.

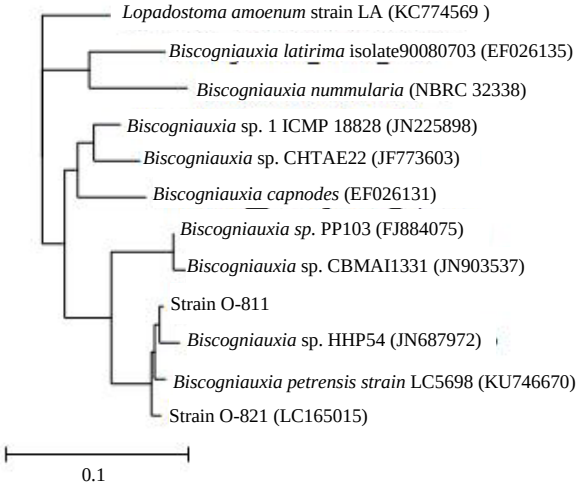

Fig. 3 Phylogenetic tree based on ITS sequences of strain O-811. A bootstrap consensus neighbor-joining tree for the strain O-811 was created based on the Kimura 2-Parameter distance matrix (1000 replicates). Lopadostoma amoenum strain LA (KC774569) was used as the out-group. The scale bar represents 10% sequence dissimilarity.

For the detection of the inhibitory compound of O-811-ECF, the growth inhibition zone against *M. oryzae* was investigated using the TLC plates. The authentic HDFO was used as a control. In O-811-ECF, the growth inhibition zone was observed at Rf of 0.66 (Fig. 4). Furthermore, the growth inhibition zone by HDFO was observed at Rf of 0.66. These results suggest that the inhibitory compound included in strain O-811 isolate of *Biscogniauxia* sp. is the same as the one from HDFO of strain O-821.

The HDFO adjusted from O-821-ECF exhibited inhibitory activity against *M. oryzae* at <5 ppm. In addition, HDFO exhibited inhibitory activity against *Alternaria alternata* Japanese pear pathotype, *Cochliobolus miyabeanus, Colletotrichum orbiculare, Corynespora cassiicola, Fusarium oxysporum* f. sp. *conglutinans*, and *F. oxysporum* f. sp. *spinaciae* at >50 ppm (Nguyen et al., 2018). Therefore, it is necessary to investigate whether O-811-ECF exhibits inhibitory activity against other plant pathogens.

Interestingly, Nguyen et al. (2017) reported that O-821-ECF induced resistance against *M. oryzae* in rice. Therefore, it is also necessary to investigate whether O-811-ECF induces resistance, as well as to determine its inhibitory mechanism.

In conclusion, the study of the strain O-811 of *Biscogniauxia* may contribute to the development of a new fungicide for the control of plant diseases such as the rice blast disease.

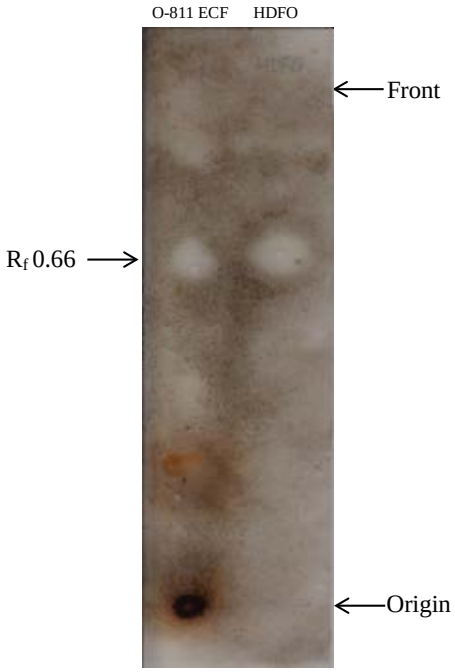

Fig. 4 Thin layer chromatography (TLC)-profiles of the ethyl acetate extracts of culture filtrate of the O-811 isolate (O-811 ECF). O-811 isolate ECF was spotted onto the TLC plate. After development, the TLC plate was sprayed with a concentrated conidia suspension of *Magnaporthe oryzae* in the presence of potato sucrose agar. Inoculated plates were kept in a moist chamber at 26–28 ℃ for 3 d. HDFO: (3aS,4aR,8aS,9aR)-3a-hydroxy-8a-methyl-3,5-dimethylenedecahydronaphto[2,3-b]furan-2(3H)-one

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