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Inhibition of *Magnaporthe oryzae* by Culture Filtrates of Fungi Isolated from Wild Mushrooms

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Abstract

Fungal strains isolated from the fruiting bodies of wild mushrooms were evaluated for fungicidal activity against *Magnaporthe oryzae*, the causal agent of the rice blast disease. Fungal isolates (n = 105) were obtained from 46 samples of wild mushrooms. Infection behaviors of *M. oryzae* were assessed in the presence of culture filtrates from 90 fungal isolates, of which 20 inhibited spore germination. Heat-treated culture filtrates of these isolates were classified into 3 groups according to biological activity. Blast lesion formation by *M. oryzae* was significantly inhibited by pretreatment with culture filtrates from 4 fungal isolates. ITS region sequence analysis indicated that these isolates shared similarities with species of the genera *Annulohypoxylon*, *Nigrospora*, and *Penicillium.* Studies of symbiotic and parasitic fungi from wild mushrooms may yield potential control agents for plant diseases such as the rice blast disease.

Keywords

1. Introduction

Rice is one of the world's most important food crops and the rice blast disease caused by *Magnaporthe oryzae* (Hebert) Barr is the most destructive fungal disease affecting global rice production. Also, *M. oryzae* causes blast disease on several grass species including the cereals wheat and barley.

Resistant rice cultivars and chemical fungicides are important in blast disease control. However, the durability of genetic resistance in improved rice cultivars is often short-lived in the field because the pathogen rapidly evolves to overcome resistance [1]. The key to chemical control of rice blast disease is to overcome fungicide-

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resistant pathogens [2] [3]. Therefore, a search for antifungal compounds is required to develop new fungicides. Antifungal compounds of microbial origin play an important role in the biological and chemical control of plant diseases [4]-[6].

Some phylloplane and endophytic fungi protect plants from pathogens. These fungi produce antimicrobial compounds [7]-[9]. Kawamata *et al.* (2004) [10] reported that fungal species isolated from the phylloplane of rice plants inhibit not only *M. oryzae* conidium germination and mycelial growth but also blast lesion formation. Furthermore, *Pseudomonas aeruginosa* (Schroeter) Migula isolated from spent mushroom substrate significantly inhibits *Pyricularia grisea* (Cooke) Sacc, the causal agent of gray leaf spot in perennial ryegrass [11].

The number of mushroom species in the world is estimated to be about 140,000, but only 10% have been identified [12]. There are no reports on the existence of symbiotic and parasitic fungi in wild mushrooms and their utility in pathogen control. The objectives of this study were to: 1) isolate symbiotic and parasitic fungi from the fruiting bodies of wild mushrooms; 2) investigate the antifungal activity of culture filtrates of these fungi against *M. oryzae*; and 3) investigate the protective effect of the culture filtrates of the fungal isolates on lesion formation by *M. oryzae* in barley leaves.

2. Materials and Methods

2.1. Isolation of Fungi from Fresh Fruiting Bodies of Wild Mushrooms

Fungi were isolated from fresh fruiting bodies of 46 wild mushroom samples collected from a paddy field in Fukui, Hiroshima, Kumamoto, Osaka, Shimane, and Tottori prefectures in 2009-2011. Each fresh fruiting body was cut with a blade. Segments (approx. 5×5 mm) from each fresh fruiting body were removed and surface-sterilized in 80% ethanol for 2 min and 1% sodium hypochlorite solution for 3 min, then rinsed twice in sterile distilled water for 2 min. Rinsed segments were placed on sterile filter paper in petri dishes to remove excess surface water. After drying the segments, each segment was placed on potato sucrose agar (PSA) medium containing chloramphenicol (200 µg/mL) in petri dishes (9 cm diameter) and incubated for 14 days at 27˚C. Fungal isolates that grew on the segments were transferred to fresh PSA medium and fungal isolates obtains by single spore or colony was maintained on PSA slants until use.

2.2. Preparation of the Culture Filtrate

Mycelial disks (7 mm diameter) prepared from a single colony of each isolate grown on PSA medium for 7 days were incubated in a test tube containing 20 mL potato sucrose broth (PSB). The culture was kept in the dark condition at 27˚C and shaken at 120 rpm for 10 days using rotary shaker. Culture filtrates were collected through a 0.2-µm membrane filter.

2.3. Infection-Inhibiting Activity of the Culture Filtrates

Magnaporthe oryzae (strain Naga 69 - 150, race 007) was grown on rice bran agar at 26˚C for 14 days, washed with running water to remove aerial hyphae, and kept at 26˚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) were suspended in the fungal culture filtrates, distilled water or PSB were dropped onto glass slides and kept in a moist chamber at 26˚C. After 24 h incubation, the percentage of spore germination was determined by light microscopy. Data analyses were performed by using the Scheffe's test.

2.4. Heat Pretreatment

Selected culture filtrates were heated at 121˚C for 20 min using the autoclave. The treated and untreated filtrates were inoculated together with a spore suspension (1×10^5 spores/mL) of *M. oryzae* onto glass slides and kept in a moist chamber at 26˚C. After 24 h incubation, the percentages of spore germination and appressorium formation were determined by light microscopy. Data analyses were performed by using the Scheffe's test.

2.5. Culture Filtrate Pretreatment and Fungal Inoculation

To investigate the effects of culture filtrates on the induction of blast resistance, barley seedlings (*Hordeumvul-

gare L., cv. Minoriomugi) at the 1- to 2-leaf stage were sprayed with culture filtrates. PSB and distilled water spray were used as a control. The pretreated barley plants were kept under light condition in a moist chamber at 26˚C for 24 h, and then sprayed with a suspension of *M. oryzae* spores (1×10^5 spores/mL). Inoculated plants were incubated in a moist chamber at 26˚C. Blast lesions on barley leaves were counted 4 days after inoculation.

2.6. DNA Extraction and PCR Amplification and Sequencing

To identify the active isolate, 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'-TCCTCCGCT TATTGATATGC-3') primers. Fungal genomic DNA was extracted from the mycelia as described by Suzuki *et al.* (2006) [13], 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).

2.7. Statistical Analysis

Data are reported as mean ± standard deviation. Significant differences from the values were determined using a Scheffe's test ($p < 0.05$).

3. Results and Discussion

105 fungal isolates were obtained from the 46 fruiting bodies of wild mushrooms collected. Twenty of 90 fungal culture filtrates inhibited *M. oryzae* spore germination. The percent spore germination after treatment with isolates F5, K7, K24, K36, O1, S5, and S8 was showed 1.8% ± 5.3%, 12.4% ± 21.0%, 34.7% ± 35.5%, 18.7% ± 7.3%, 2.9% ± 5.9%, 0.9% ± 2.0%, and 27.3% ± 19.2%, respectively. On the other hand, the percent spore germination of culture filtrates by using of isolates of F1, F2, F3, H2, H5, H7, H9, K1, K9, K21, O3, O6, and S9 was 0%. Germination in the distilled water and PSB controls was 95.8% ± 3.7% and 90.9% ± 5.7% (**Figure 1**).

The heated-culture filtrates (n = 20) were classified into 3 groups according to their inhibitory activity. The first group (F3, H2, K21, K24, and S8) showed no antifungal activity (**Figure 2**). These culture filtrates did not inhibit *M. oryzae* infection behaviors such as spore germination and appressorium formation. The second group (F2, F5, H9, K1, O1, O3, O6, S5, and S9) inhibited *M. oryzae* infection behaviors. The third group (F1, H5, H7, K7, K9, and K36) inhibited appressorium formation but not spore germination (**Figure 2**). Ethyl acetate extracts of culture filtrate of isolates F2, F5, H9, K1, O1, O3, O6, S5, and S9 significantly inhibited both spore germination and appressorium formation. However, F3, H2, K21, K24, and S8 did not (**Figure 2**). Some fungi produce enzymes such as chitinase and *β*-1,3-glucanase that mediate cell wall and cell membrane damage in plant pathogens [14] [15]. Our results suggested that the antifungal compound(s) in the culture filtrates of F2, F5, H9, K1, O1, O3, O6, S5, and S9 were not high-molecular-weight enzymes such as chitinase and *β*-1,3-glucanase. However, F3, H2, K21, K24, and S8 may express chitinase and *β*-1,3-glucanase. F1, H5, H7, K7, K9, and K36 may express high-molecular-weight enzymes as well as low-molecular-weight metabolites.

Barley leaves were sprayed with culture filtrates of isolates F2, F5, H9, K1, O1, O3, O6, S5, and S9, all of which show antifungal activity, and inoculated with *M. oryzae* spores 24 h later. Blast lesion formation was significantly inhibited by pretreatment with culture filtrates of O1, O3, S5, and S9 in comparison to distilled water and PSB controls. However, culture filtrates of F2, F5, H9, K1, and O6 did not inhibit blast lesion formation (**Figure 3**).

To identify the mycological characteristics of the obtained fungi, the ITS region including 5.8S rDNA was PCR-amplified from the genomic DNA of isolates O1, O3, S5, and S9 using ITS1 and ITS4 primers. The sequences shared similarity with *Annulohypoxylon* sp. (S9), *Nigrospora* sp. (S5), and *Penicillium* sp. (O1 and O3) (**Table 1**).

Penicillium spp. are known biocontrol agents with inhibitory activities against various plant pathogens. *Peni-*
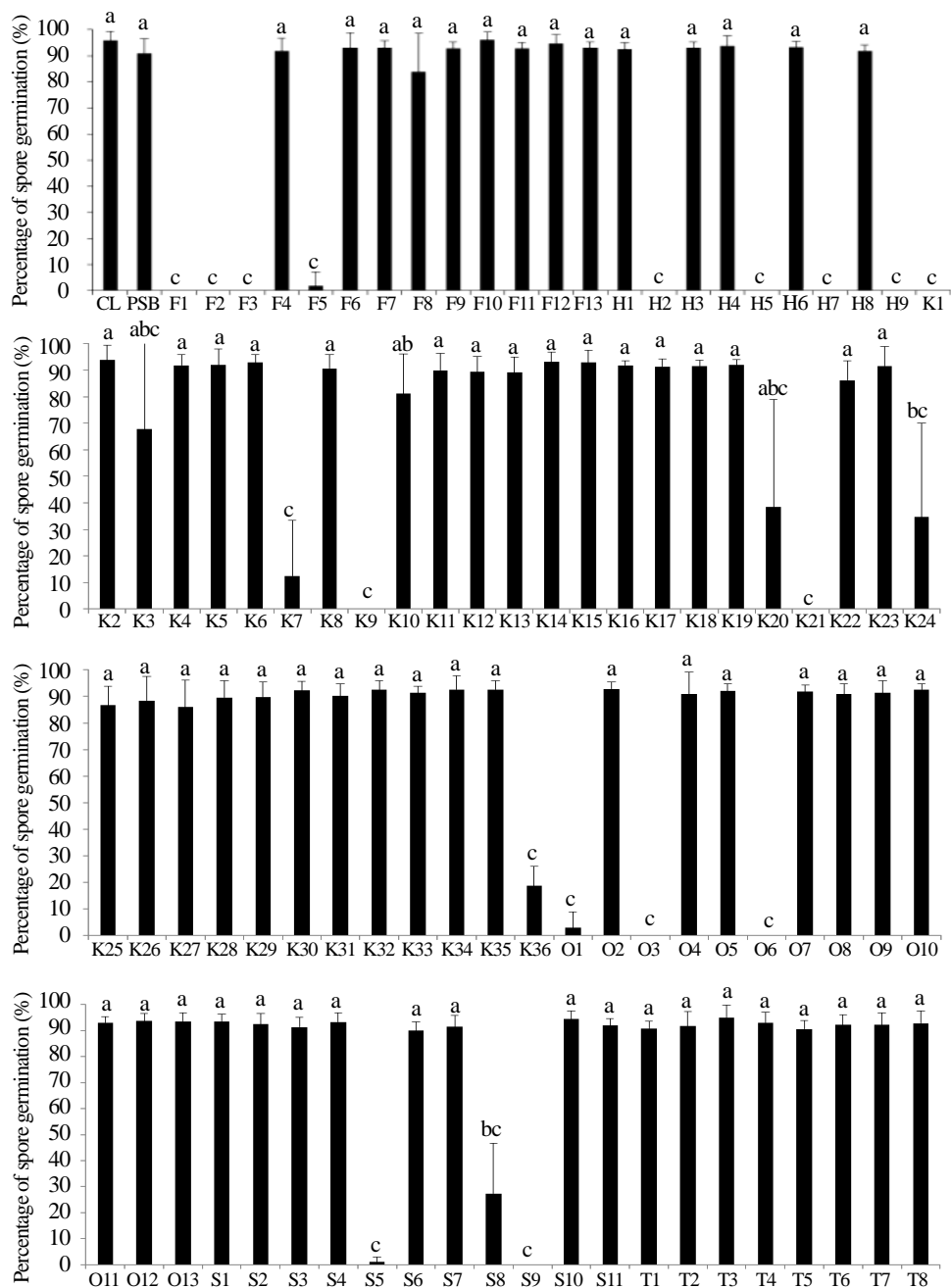
Figure 1. Culture filtrates of fungi isolated from wild mushrooms influence the infection behaviors of *Magnaporthe oryzae*. Data were obtained from the results of 6 experiments of 3 replicates and expressed as mean ± SD. Means followed by different letters are significantly different according to the Scheffe's test ($p < 0.05$).

cillium spp. produce antifungal compounds such as mycophenolic acid, patulin, 3-omethylfunicone, and decumbenone A, which directly or indirectly inhibit infection by plant pathogens [16] [17]. In addition, phomalactone isolated from *Nigrospora sphaerica* (Sacc.) E.W. Mason inhibits mycelial growth and affects the infection behavior of plant pathogens [18]. Nigrosporin A and B, which are phytotoxic metabolites, are produced from a culture filtrate of *Nigrospora oryzae* (Berk. & Br.) Petch [19]. However, in this study, the culture filtrate of *Nigrospora* sp. showed no phytotoxic activity in barley. There are no reports on the utility of culture filtrates of *Annulohypoxylon* for control of plant pathogens. *Annulohypoxylon* is members of the Xylariaceae family and can
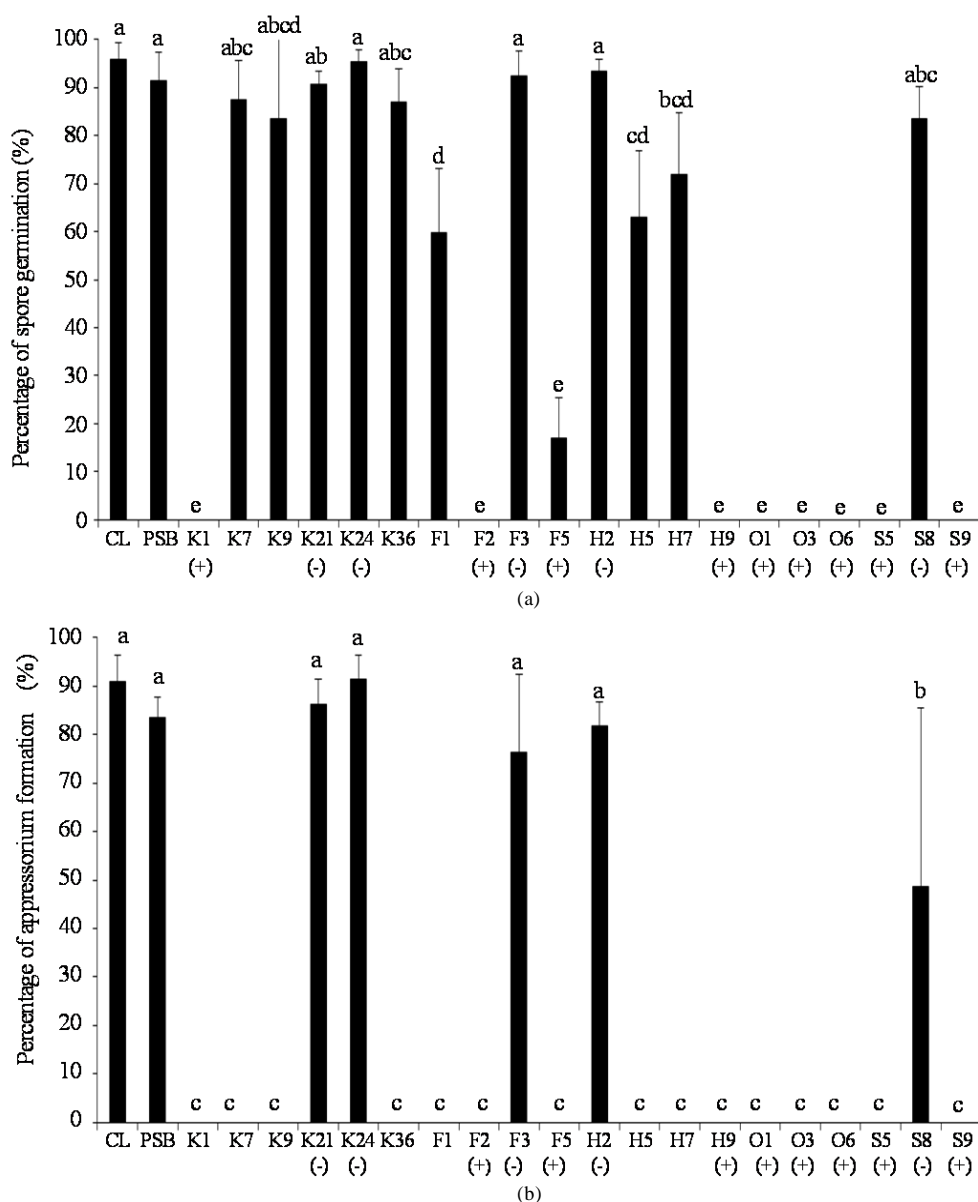
Figure 2. Inhibition by heat-treated culture isolates on the infection behaviors of *Magnaporthe oryzae*. (a) and appressorium formation (b) were determined after 24 h incubation. (+): culture filtrates with ethyl acetate soluble (+) and insoluble (−) antifungal compound against *M. oryzae* spore germination and appressorium formation. Data were obtained from the results of 6 experiments of 3 replicates and expressed as mean ± SD. Means followed by different letters are significantly different according to the Scheffe's test ($p < 0.05$).

survive as endophytes. Xylariaceae is a large family (Xylariales, Ascomycotina) of more than 36 genera. Whalley (1995) [20] reported that secondary metabolites produced by representatives at least one-third of these genera have been isolated and identified. Further studies are essential to identify the active antifungal compound(s) in the culture filtrates of these fungi. Studies of symbiotic and parasitic fungi from wild mushrooms may yield potential control agents for plant diseases such as the rice blast disease.
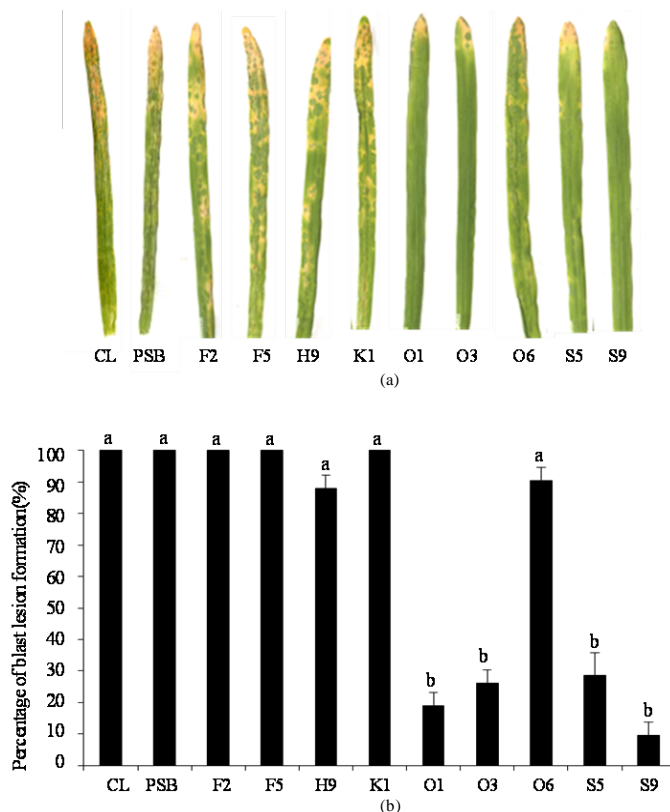
Acknowledgements

Figure 3. Inhibition by culture filtrates of selected isolates on lesion formation by *Magnaporthe oryzae* in barley leaves. (a) Blast lesion formation by *M. oryzae*. (b) Percentage of blast lesion formation by *M. oryzae*. Data were obtained from the results of 10 experiments of 3 replicates and expressed as mean ± SD. Means followed by different letters are significantly different according to the Scheffe's test ($p < 0.05$).

Table 1. Identification of fungi isolated from mushroom by sequences of rDNA ITS regions.

Isolate	Candidate fungus	Sampling location	Sampling year
O1	*Penicillium* sp.	Japan (Osaka)	2010
O3	*Penicillium* sp.	Japan (Osaka)	2010
S5	*Nigrospora* sp.	Japan (Shimane)	2009
S9	*Annulohypoxylon* sp.	Japan (Shimane)	2009

supports for publishing this report.

References

[1] Ahn, S.W. (1994) International Collaboration on Breeding for Resistance to Rice Blast. In: Zeigler, R.S., Leong, S.A. and Teng, P.S., Eds., *Rice Blast Disease*, CAB International, Wallingford, 137-153.

[2] So, K., Fuji, M., Iwabuchi, H., Kanayama, M. and Yamaguchi, J. (2002) Effects of Various Fungicides against Less Carpropamid-Sensitive Rice Blast Fungus Isolated from the Northwest Area in Saga Prefecture. *Japanese Journal of Phytopathology*, **68**, 262. (Abstract, in Japanese)

[3] Yamaguchi, J., Kuchiki, F., Hirayae, K. and So, K. (2002) Decreased Effect of Carpropamid for Rice Blast Control in the West North Area of Saga Prefecture in 2001. *Japanese Journal of Phytopathology*, **68**, 261. (Abstract, in Japanese)

[4] Fravel, D.R. (1988) Role of Antibiosis in the Biocontrol of Plant Diseases. *Annual Review of Phytopathology*, **26**, 75-

91. http://dx.doi.org/10.1146/annurev.py.26.090188.000451

[5] Shimizu, M., Nakagawa, Y., Sato, Y., Furuma, T., Igaroshi, Y., Onaka, H., Yoshida, R. and Kunoh, H. (2000) Studies on Endophyticactinomycetes and *Streptomyces* sp. Isolated from Rododendron and Its Antifungal Activity. *Journal of General Plant Patholology*, **66**, 360-366. http://dx.doi.org/10.1007/PL00012978

[6] Uddin, W. and Viji, G. (2002) Biological Control of Turfgrass Disease. In: Gnanamanickam, S.S., Ed., *Biological Control of Crop Disease*, CRC Press, Marcel Dekker, New York, 313-337.

[7] Aremu, E.A., Furumai, T., Igarashi, Y., Sato, Y., Akamatsu, H., Kodama, M. and Otani, H. (2003) Specific Inhibition of Spore Germination of *Alternaria brassicicola* by Fistupyrone from *Streptomyces* sp. TP-A0569. *Journal of General Plant Pathology*, **69**, 211-217. http://dx.doi.org/10.1007/s10327-003-0038-y

[8] Evueh, G.A. and Ogbebor, N.O. (2008) Use of Phylloplane Fungi as Biocontrol Agent against *Colletotrichum* Leaf Disease of Rubber (*Heveabrasiliensis* Muell. Arg.). *African Journal of Biotechnology*, **15**, 2569-2572.

[9] Koitabashi, M., Kajitani, Y. and Hirashima, K. (2004) Antifungal Substances Produced by Fungal Strain Kyu-W63 from Wheat Leaf and Its Taxonomic Position. *Journal of General Plant Pathology*, **70**, 124-130. http://dx.doi.org/10.1007/s10327-003-0095-2

[10] Kawamata, H., Narisawa, K. and Hashiba, T. (2004) Suppression of Rice Blast by Phylloplane Fungi Isolated from Rice Plants. *Journal of General Plant Pathology*, **70**, 131-138. http://dx.doi.org/10.1007/s10327-003-0100-9

[11] Viji, G., Uddin, W. and Romaine, C.P. (2003) Suppression of Gray Leaf Spot (Blast) of Perennial Ryegrass Turf by *Pseudomonas aeruginosa* from Spent Mushroom Substrate. *Biological Control*, **26**, 233-243. http://dx.doi.org/10.1016/S1049-9644(02)00170-6

[12] Wasser, S.P. (2002) Medicinal Mushrooms as a Source of Antitumor and Immunomodulating Polysaccharides. *Applied Microbiology and Biotechnology*, **60**, 258-274. http://dx.doi.org/10.1007/s00253-002-1076-7

[13] Suzuki, S., Taketani, H., Kusumoto, K.I. and Kashiwagi, Y. (2006) High-Throughput Genotyping of Filamentous Fungus *Aspergillus oryzae* Based on Colony Direct Polymerase Chain Reaction. *Journal of Bioscience and Bioengineering*, **102**, 572-574. http://dx.doi.org/10.1263/jbb.102.572

[14] Lorito, M., Peterbauer, C., Hayes, C.K. and Harman, G.E. (1994) Synergistic Interaction between Fungal Cell Wall Degrading Enzymes and Different Antifungal Compounds Enhances Inhibition of Spore Germination. *Microbiology*, **140**, 623-629. http://dx.doi.org/10.1099/00221287-140-3-623

[15] Shoresh, M. and Harman, G.H. (2010) Differential Expression of Maize Chitinases in the Presence or Absence of *Trichoderma harzianum* Strain T22 and Indications of a Novel Exo-Endo-Heterodimeric Chitinase Activity. *BMC Plant Biology*, **10**, 136-146. http://dx.doi.org/10.1016/S0031-9422(02)00196-6

[16] Fujii, Y., Asahara, M., Ichinose, M. and Nakajima, H. (2002) Fungal Melanin Inhibitor and Related Compounds from *Penicillium decumbens*. *Phytochemistry*, **60**, 703-708. http://dx.doi.org/10.1016/S0031-9422(02)00196-6

[17] Nicoletti, R. and Stefano, M.D. (2012) *Penicillium restrictum* as an Antagonist of Plant Pathogenic Fungi. *Dynamic Biochemistry Process Biotechnology and Molecular Biology*, **6**, 61-69.

[18] Kim, J.C., Choi, G.J., Park, J.H., Kim, H.T. and Cho, K.Y. (2001) Activity against Plant Pathogenic Fungi of Phomalactone Isolated from *Nigrospora sphaerica*. *Pest Management Science*, **57**, 554-559. http://dx.doi.org/10.1002/ps.318

[19] Tanaka, M., Fukushima, T., Tsujino, Y. and Fujimori, T. (1997) Nigrosporin A and B, New Phytotoxic and Antibacterial Metabolities Produced by a Fungus *Nigrospora oryzae*. *Bioscience Biotechnology*, *and Biochemistry*, **61**, 1848-1852. http://dx.doi.org/10.1271/bbb.61.1848

[20] Whalley, A.J.S. and Edwards, R.L. (1995) Secondary Metabolites and Systematic Arrangement within the Xylariaceae. *Canadian Journal of Botany*, **73**, 802-810. http://dx.doi.org/10.1139/b95-325