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Isolation, Cultivation, and Antifungal Activity of a Lichen-Forming Fungus

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A lichen-forming fungus was successfully isolated by discharged spore method from Korean lichen (*Heterodermia* sp.) and cultivated in pure culture. The isolate JR0012 inhibited mycelial growth of several plant-pathogenic fungi. Mycelial growth of the four *Pythium* spp. tested was completely inhibited. Potato dextrose broth was found to be the medium favorable for large-scale production of antibiotics from the isolate. Antifungal substances produced in axenic culture were partially purified. This is the first report in Korea of lichen-forming fungus successfully isolated and which exhibited strong antifungal activity against plant-pathogenic fungi, especially the four *Pythium* spp..

Keywords **:** antifungal activity, discharged spore method, *Heterodermia* sp., lichen-forming fungus, *Pythium* sp.

Lichen is one of the most widely distributed eucaryotic organisms in the world. There are about 13,500 known lichen species, which account for approximately 20% of all the fungi described (Hawksworth, 1988). These lichen species were identified based on the symbiotic association between two unrelated organisms – a fungus and an alga (or cyanobacterium). Lichen-forming fungi (LFF) are difficult to isolate and cultivate in pure culture. LFF produce a wide range of natural products, among which approximately 350 secondary metabolites have been identified (Elix et al., 1984; Galun and Shomer-Ilan, 1988). Many of these are unique to lichens and considerable numbers have been shown to have antimicrobial activity (Lawrey, 1986) or other biological activities of potential economic value (Higuchi et al., 1993; Nishitoba et al., 1987). LFF have been shown to retain in axenic cultures the capacity to biosynthesize secondary products found in the lichenized state (Culberson et al., 1992; Leuckert et al., 1990), although the metabolites produced in the greatest abundance might differ from those found in the lichen (Hamada, 1993; Miyagawa et al., 1993).

Current practices for controlling plant diseases are based largely on genetic resistance in host plant, management of the plant and its environment, and synthetic pesticides (Strange, 1993). There is a demand for new methods to supplement existing disease control strategies to achieve better disease control. Moreover, alternatives to many of the synthetic pesticides currently in use are needed. Many of these synthetic pesticides may lose their usefulness due to revised safety regulations (Benbrook et al., 1996), concern over non-target effects (Dernoeden and McIntosh, 1991), or development of resistance in pathogen populations (Russell, 1995). Thus, there is a need for new solutions to plant disease problems that provide effective control while minimizing negative consequences for human health and the environment (Cook et al., 1996). So far, little attempt has been made on the antagonistic fungi associated with Korean lichens (Hur et al., 1999). In this study, isolation and cultivation of LFF from Korean lichen (*Heterodermia* sp.) were attempted. Antifungal activity of the LFF isolate was also examined against plant-pathogenic fungi to develop less harmful and safe protectants as agrochemicals.

Lichens (*Heterodermia* sp.) growing on barks were collected in Jiri mountain, Korea, during summer. The samples were air-dried for 1 week and stored at room temperature. Identification of the lichens was carried out according to the classifications of Park (1990) and Yoshimura (1994) before the isolation. Lichen-forming fungi were isolated by discharged spore method (Crittenden et al., 1995). Thalli bearing fruiting bodies were washed for 1 hour in a turbulent flow of tap water in order to remove as much surface contamination as possible. Either individual ascomata or fragments of thallus bearing many small ascomata were cut off and attached to the inside of Petri dish lids with petroleum jelly. Petri dishes containing agar were then inverted over the lids and the ascospores were allowed to discharge upwards onto the agar medium. Cultures were incubated at 20°C in the dark and examined periodically during a 1-month period. Germinating spores were transferred to fresh medium. Mycobiont isolate

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(JR0012) produced a compact mycelium 5-10 mm in diameter 1 or 2 months after incubation, and subcultured onto fresh medium for long-term storage. The culture medium of Crittenden et al. (1995) was routinely used for isolation and growth of LFF.

A mycelial disc (1 cm in diameter) cut from the margin of actively growing cultures of the isolate JR0012 was inoculated into a 500 ml Erlenmeyer flask containing 200 ml of potato dextrose broth (PDB, Difco, USA). After 10 days of incubation at 20°C and 150 rpm, liquid culture was filtered through a Whatman No. 1 paper. The filtrate was evaporated under reduced pressure at a temperature not exceeding 55°C. The concentrated filtrate was suspended in 10 ml of MeOH. Antifungal activity of the MeOH extract was evaluated against several plant-pathogenic fungi by agar well method. Single well (8 mm in diameter) was prepared at the center of the potato dextrose agar plate. Sterile filter paper (8 mm in diameter) was placed at the bottom of the well and 200 μl of the MeOH extract or MeOH (control) was dripped into the well and then left in a clean bench until the solvent was completely dried. Agar plug (8 mm in diameter) cut from the margin of actively growing cultures of the plant-pathogenic fungi was placed on the filter paper in the well. Inhibition of fungal growth was examined by measuring the diameter of mycelium mat on the plate after 5-10 days of incubation at 25°C.

To select the medium favorable for large-scale production of antibiotics from LFF JR0012 isolate, six media consisting of various nutritional elements were tested. The six media used in this study were as follows: Czapek-Dox medium, Elliott's medium, Fries medium, Malt and yeast extract medium, Potato dextrose broth (Difco, USA), and Crittenden & Oliver's defined medium (Crittenden et al., 1995). The isolate was cultivated in a 500 ml Erlenmeyer flask containing 200 ml of the six media, respectively. After 10 days of incubation at 20°C and 150 rpm, mycelial mats were harvested, dried at 60°C for 24 hours, and dry weight was measured to compare fungal growth in each medium. Antifungal activity of the concentrated filtrates was evaluated against *Pythium graminicola* by agar well method as mentioned above.

Five mycelial discs (1 cm in diameter) cut from the

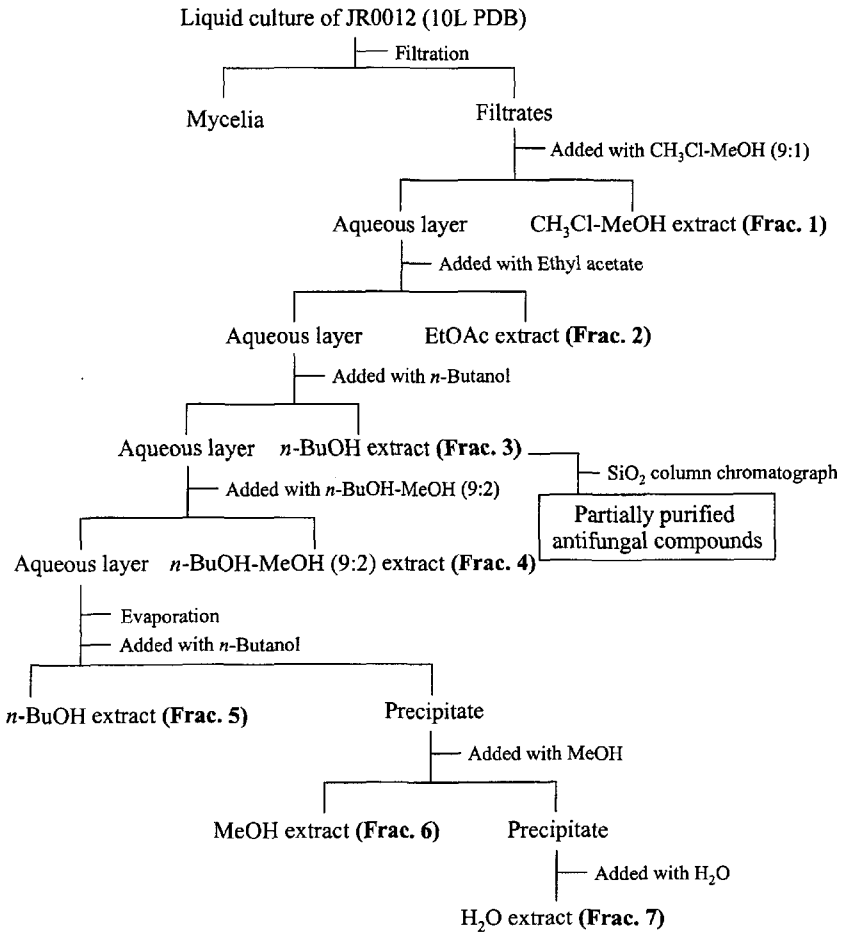

Fig. 1. Successive partition of liquid culture of LFF isolate JR0012 and partial purification of antifungal compounds.

Table 1. Antifungal activity of lichen-forming fungus (JR0012) isolated from lichen (*Heterodermia* sp.) against several plant-pathogenic fungi

Test fungus	Diameter of mycelial mats (mm)		Inhibition rate[a] (%)
	JR0012	Control	
Bipolaris coicis	45.76 ± 0.35	64.10 ± 2.46	–
Botryosphareia dothidea	34.30 ± 2.20	36.80 ± 0.90	6.8
Botrytis cinerea	65.20 ± 2.90	67.50 ± 2.30	3.4
Cercospora kikuchii	42.22 ± 0.71	45.29 ± 0.88	6.8
Collectotricum coccodes	19.10 ± 0.23	21.55 ± 0.28	11.4
Collectotricum gloeosporioides	39.73 ± 0.16	47.32 ± 0.62	16.0
Collectotricum orbiculare	23.50 ± 0.35	24.03 ± 0.57	2.2
Fusarium graminearum	53.85 ± 0.44	59.67 ± 0.23	9.8
Magnaporthe grisea	49.20 ± 1.99	58.60 ± 1.49	16.0
Pestalotiopsis longiseti	53.78 ± 1.76	53.62 ± 1.45	–
Phomopsis mali	50.30 ± 0.47	51.30 ± 2.90	2.0
Phomopsis soje	26.39 ± 2.37	32.93 ± 2.45	19.9
Pythium aphanidermatum	–	90.00	100.0
Pythium graminicola	–	90.00	100.0
Pythium ultimum var. *ultimum*	–	90.00	100.0
Pythium	–	90.00	100.0
Rhizoctonia solani	42.64 ± 3.12	59.86 ± 2.09	28.8
Sclerotinia sclerotiorum	63.31 ± 3.55	66.03 ± 0.69	4.1

[a] Inhibition rates were calculated by the following equation: (1–dt/dc) × 100, where dt is the average diameter of fungal colony treated with the extracts (JR0012) and dc is the average diameter of fungal colony treated with MeOH (Control). Each value represents a mean of five replicates.

Table 2. Mycelial growth and antifungal activity of LFF isolate JR0012 in several media

Media	Mycelial growth (g day weight/100 ml)	Inhibition rate (%) of mycelial growth[a]
Czapek-Dox medium	0.35 ± 0.07	22.7
Elliott's medium	0.16 ± 0.03	18.5
Fries medium	0.12 ± 0.05	58.9
Malt & yeast extract medium	0.21 ± 0.02	28.4
Potato dextrose broth	0.35 ± 0.05	100.0
Crittenden & Oliver's medium	0.03 ± 0.02	38.9

[a] Antifungal activity was evaluated against *Pythium graminicola* by agar well method in Table 1.

margin of actively growing cultures of the isolate was inoculated in 10 L of potato dextrose broth (PDB). After 10 days of incubation at 20°C on a rotary shaker (150 rpm), 8.5 L of filtrate was used. The antibiotic substances produced by the isolate were partially purified as described in Fig. 1 and antagonistic activity of each fraction was investigated by agar well method as mentioned above.

The JR0012 was successfully isolated from lichen (*Heterodermia* sp.) by discharged spore method and showed antagonistic activity against several plant-pathogenic fungi (Table 1). While mycelial growth of the other fungal pathogens was not consistently inhibited, all the *Pythium* species tested were completely inhibited.

Among the six media tested, PDB was selected as the most favorable for large-scale production of antibiotics from the isolate. Mycelial growth and antifungal activity was higher in PDB than in other media (Table 2). Liquid culture of the isolate was separated by using different solvents as described in Fig. 1, and the resulting fractions were examined to determine the antifungal activity against *Pythium graminicola*. Among the fractions, *n*-BuOH fraction (Frac. 3) exhibited complete inhibition of mycelial growth (data not shown). The antifungal-active fraction (Frac. 3) was chromatographed on a SiO_2 column with step-gradient elution of CH_3Cl-MeOH-H_2O and partially purified. Further purification of antifungal substances is now ongoing.

Preservation ethics cause many problems in developing novel antifungal compounds from intact lichens. Lichens grow very slowly, and an entire population can be removed from an area during the collection. A great amount of lichen

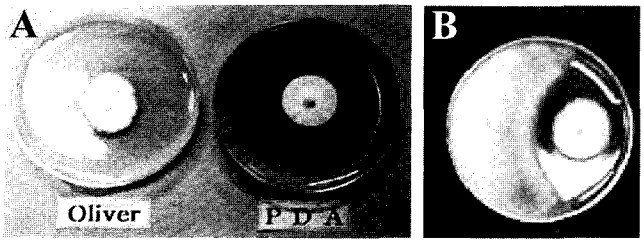

Fig. 2. Lichen-forming fungus (JR0012 isolate) isolated from *Heterodermia* sp. lichen. (**A**) Colony of the isolate on potato dextrose agar (PDA) incubated at 20°C for 30 days or Crittenden & Oliver's defined medium (Oliver) incubated at 20°C for 50 days. (**B**) Antifungal activity of the isolate against *Pythium graminicola* on PDA. The fungal pathogen was inoculated 30 days after inoculation of the isolate and incubated at 25°C for 3 days.

materials is also needed for purification and chemical identification of biologically active compounds. For these reasons, use of lichen-forming fungi is an alternative means to develop natural products having antimicrobial activity or other biological activities of potential economic value. Lichenicolus fungal isolate showing antifungal activity against several plant-pathogenic fungi was previously reported (Hur et al., 1999). However, this is the first report in Korea of lichen-forming fungus with strong antifungal activity successfully isolated by discharged spore method and cultivated in pure culture. Furthermore, antifungal substances produced by the isolate in axenic culture were specifically active against four *Pythium* spp.. Further research on purification, identification of antifungal molecules, and evaluation of their antifungal activity specific to the *Pythium* spp. is in progress.

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