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Note

Antifungal Activity of Lichen-Forming Fungi Isolated from Korean and Chinese Lichen Species Against Plant Pathogenic Fungi

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Antifungal activity of Korean and Chinese lichen-forming fungi (LFF) was evaluated against plant pathogenic fungi of *Botryosphaeria dothidea*, *Botrytis cinerea*, *Diaporthe actinidiae*, *Pestalotiopsis longiseta*, *Pythium* sp., *Rhizoctonia solani*, and *Sclerotium cepivorum*. The LFF were isolated from *Cladonia scabriuscula*, *Melanelia* sp., *Nephromopsis asahinae*, *Nephromopsis pallescens*, *Parmelia laevior*, *Pertusaria* sp., *Ramalina conduplicans*, *Ramalina sinensis*, *Ramalina* sp., *Umbilicaria proboscidea* and *Vulpicida* sp. with discharged spore method. The isolates were deposited in the herbarium of Korean Lichen Research Institute (KoLRI) in Sunchon National University. The LFF of *Melanelia* sp., *P. laevior*, *Pertusaria* sp., *R. conduplican* and *Ramalina* sp. exhibited strong antifungal activity against all of the pathogenic fungi examined. Among them, LFF of *P. laevior* showed more than 90% of inhibition in fungal hyphae growth, compared with control. The results imply that LFF can be served as a promising bioresource to develop novel biofungicides. Mass cultivation of the LFF is now under progress in laboratory conditions for chemical identification of antifungal substances.

Keywords **:** biofungicide, discharged spore method, lichen-forming fungi, novel bioresource, *Parmelia laevior*

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 the synthetic pesticides in these days 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).

Lichens are symbiotic organisms composed of a fungus (mycobiont) and an algae (photobiont). They produce characteristic secondary metabolites, lichen substances, which seldom occur in other organisms. Lichen and their metabolites have various biological activities such as antimicrobial, antifungal, antiviral, antiprotizoal, antiproliferative, antioxidant and anti-inflammatory (Behera et al., 2005; Halama and Van Haluwin, 2004; Ingolfsdottir, 2002; Müller, 2001; Perry et al., 1999; Yamamoto et al., 1998). In spite of the wide spectrum of biological activities shown by the lichens, they have long been neglected by mycologists and overlooked by agrochemical industry because of their slow growth in nature and difficulties in the artificial cultivation of organisms. Hence the large-scale industrial production of the lichen metabolites has never been accomplished. However, use of lichen-forming fungi (LFF) can overcome the disadvantage of natural lichen extracts for industrialization of their metabolites because of their much faster growth and larger production of the metabolites in culture than the natural thalli.

Until now, little attempt has been made to evaluate antifungal activity of LFF isolated from lichens against plant pathogenic fungi to develop less harmful and safe protectants as novel agrochemicals. In this study, isolation of LFF was attempted from Korean and Chinese lichen materials. Antifungal activity of the isolates was also screened to provide novel bioresources necessary for biofungicide industrialization.

The natural thalli of Korean lichens were collected from 20 mountain areas in South Korea during 2002 to 2004 (Hur et al., 2004) and used in this study mostly. Chinese lichens were also collected from highland areas of Yunnan Province, China (Hur et al., 2005). Identification of Korean and Chinese lichens were also described in the above publications. Lichen specimens were air-dried for 1 week at room temperature and stored -20°C until isolation of lichen-forming fungi (mycobints). All of the lichen materials

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examined were deposited at the herbarium of Korean Lichen Research Institute, Sunchon National Univeristy, Korea (http:/lichen.sunchon.ac.kr).

The colonies of LFF were obtained using discharged spore method (Yoshimura et al., 2002). Thalli bearing fruiting bodies were washed for 1 h in a turbulent flow of tap water in order to remove surface contamination as much as possible. Either individual ascomata or fragments of thallus bearing many small ascomata were cut off and attached with petroleum jelly to the inside of Petri dish lids. Petri dishes containing malt-yeast extract medium were then inverted over the lids and the ascospores allowed to discharge upwards onto the medium. Cultures were incubated at 18°C in the dark and examined periodically during 1-month period. Germinating spores were transferred to fresh medium. Mycobiont isolates produced a compact mycelium of with 5-10 mm in diameter for 5 month after incubation and subcultured onto fresh medium for further study. The culture medium of malt-yeast extract was routinely used for isolation and growth of LFF (Yoshimura et al., 2002).

Freshly grown two mycelial masses (3 mm diam.) of the isolated LFF were placed at the edge of malt-yeast extract plate (6 cm diam.) at same distance from the plate center. Due to slow growth of LFF, the isolates were incubated on the agar medium at 18°C in dark condition for 60 days before inoculation of plant pathogenic fungi. Freshly grown mycelial agar block (3 mm diam.) of *Botryosphaeria dothidea*, *Botrytis cinerea*, *Diaporthe actinidiae*, *Pestalotiopsis longiseta*, *Pythium* sp., *Rhizoctonia solani* and *Sclerotium cepivorum* was placed on the center of the pre-incubated agar plate. The inhibition zone of mycelial growth of the pathogenic fungi was rated 3 to 5 days after incubation at 18°C and compared with the control plate.

Total 12 lichen-forming fungal isolates were obtained from Korean and Chinese lichens using discharged spore method (Fig. 1 & Table 1). Isolation of LFF from *Nephromopsis asahinae* and *N. pallescence* lichen species were firstly reported in this study (Crittenden et al., 1995; Yamamoto, 2002; Yoshimuara et al., 2002). All of the isolates were quite different from normal fungi in morphology. Their mycelial masses were enlarged as a callus-like aggregation with very short hairy hyphae on the mass. Some isolates leached large amount of pigments into the agar medium. This suggested that secondary metabolites of LFF were produced and diffused into the medium. Compared with normal fungi, LFF grew very slowly and developed less than 1 cm diameter of mycelium mass within 5 months. However, the growth rate can be considered to be much faster than that of the natural lichen thalli (Yamamoto et al., 1993).

The isolates of LFF showed very strong antifungal activity against several plant pathogenic fungi (Table 2). Among the

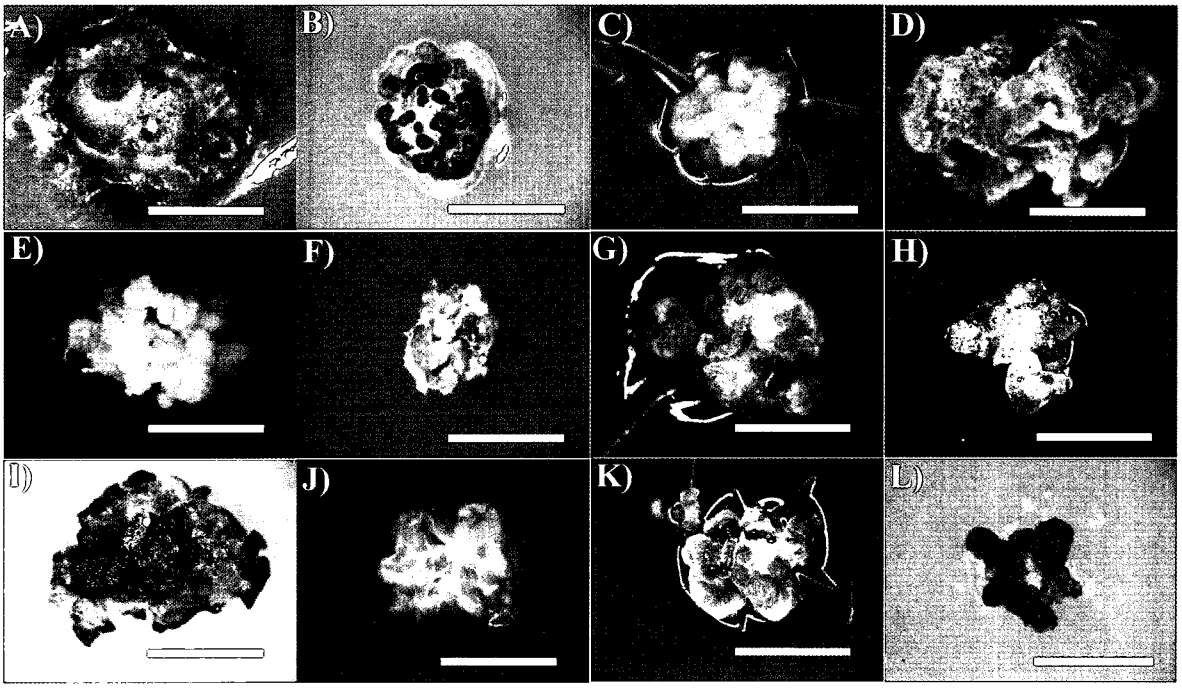

Fig. 1. Lichen-forming fungi (LFF) isolated from Korean and Chinese lichens by discharged spore method. The isolates were cultivated on malt-yeast extract medium at 18°C in dark condition for 5 months (Bar = 5 mm). A) *Cladonia scabriuscula*, B) *Melanelia* sp., C) *Nephromopsis asahinae*, D) *Nephromopsis pallescens*, E) *Parmelia laevior*, F) *Pertusaria* sp., G) *Ramalina conduplicans*, H) *Ramalina sinensis*, I) *Ramalina* sp., J) *Ramalina* sp. K) *Umbilicaria proboscidea* and L) *Vulpicida* sp.

Table 1. Lichen-forming fungi isolated from the ascospore of Korean and Chinese lichens and their morphological and cultural characteristics. All isolates were cultured on malt-yeast extract medium at 18°C in dark condition for 5 months

Lichen species	Collection number	Place	Hairy hyphae	Mycelium color	Pigmentation of medium	Relative growth rate
Cladonia scabriuscula	040481	Mt. Odae, Korea	+	brown	brown	+++
Melanelia sp.	ChyOp0406	China	+	dark brown	–	++
Nephromopsis asahinae	040500	Mt. Odae, Korea	+	pale pink	–	++
Nephromopsis pallescence	040516	Mt. Odae, Korea	+	pale brown	–	+++
Parmelia laevior	040257	Mt. Jiri, Korea	–	pale yellow	–	+++
Pertusaria sp.	041303	Mt. Seolark, Korea	+	brown	–	+
Ramalina conduplicans	040402	Mt. Odae, Korea	+	dark gray	dark brown	+++
Ramalina sinensis	Chy04020	China	+	dark gray	brown	++
Ramalina sp.	041301	Mt. Seolark, Korea	+	brown	–	+++
Ramalina sp.	Op04012	Mt. Jiri, Korea	+	pink	–	++
Umbilicaria proboscidea	Chy04077	China	+	dark gray	dark brown	++
Vulpicida sp.	chyOp0404	China	–	brown	–	+

+ : mycelial mass > 3 mm (in diam., 5 months incubation on malt-yeast extract), ++ : 3 < mycelial mass < 5 mm, +++ : mycelial mass > 5 mm

Table 2. Antifungal activity of lichen-forming fungal isolates (% of mycelial growth inhibition rate)

Lichen species	*Botryosphaeria dothidea*	*Botrytis cinerae*	*Diaporthe actinidiae*	*Pestalotiopsis dothidea*	*Phythium* sp.	*Rhizoctonia solani*	*Sclerotium cepivorum*
Cladonia scabriuscula	–	–	–	–	–	–	–
Melanelia sp.	100	58.1	77	58.9	100	52.5	+
Nephromopsis asahinae	–	24.7	31.2	24.8	–	–	–
Nephromopsis pallescence	55.5	–	45.2	48.2	+	43.8	24.3
Parmelia laevior	100	100	92.1	89.3	100	100	100
Pertusaria sp.	91.9	21.9	79.4	94	100	49	79.2
Ramalina conduplicans	68	100	48	19	+	–	100
Ramalina sinensis	76.1	78.4	28.3	–	+	76.9	51
Ramalina sp. (041301)	90.1	21.6	76.7	80.9	100	44.6	79.7
Ramalina sp. (Op04012)	59.2	39.8	58.6	47.5	100	–	72.5
Umbilicaria proboscidea	56.1	–	45.3	35.2	–	–	39.7
Vulpicida sp.	–	–	–	–	–	52.5	–

+ : active (inhibition rate less than 20%), –: inactive

12 isolates, LFF of *P. laevior* most effectively inhibited mycelial growth of all the test fungi. It was completely inhibited the mycelial growths of *Botryosphaeria dothidea*, *Botrytis cinerea*, *Pythium* sp., *Rhizoctonia solani* and *Sclerotium cepivorum* (Fig. 2). LFF of *Melanelia* sp., *Nephromopsis pallenscens*, *Pertusaria* sp. and *Ramalina* sp. also showed antifungal activity against all the tested fungi with inhibition rates of 20 to 100%. However, LFF of *Cladonia scabriuscula* and *Vulpicida* sp. had no detectable antifungal activity against the pathogens. In general, antifungal activity of the LFF isolates was dependent on the lichen species rather than the tested pathogenic fungal species. This implies that the isolated LFF had a broad spectrum of antifungal activity. The LFF obtained from *Ramalina* genus exhibited moderate antifungal activity against the pathogenic fungi. *Ramalina* lichen was well known to produce usnic acid which was proved to have a strong antimicrobial and antifungal activity (Ingolfsdottir, 2002).

Numerous ascospores of *Parmelia laevior* were successfully discharged from an apothecium, germinated and grown on the medium (Fig. 3B). In addition to strong antifungal activity with a broad spectrum, growth rate of *P. laevior* LFF was relatively faster among the 12 isolates (Fig. 1). *P. laevior* is one of the common lichen species in *Parmelia* genus in Korea. The lichen can be easily identified in the field due to the presence of puctate pseudocyphellae along the thallus margin (Fig. 3A) and contains atranorin and salazinic acid as main secondary metabolites (Kurogawa, 1994). Atranorin was reported to have no antifungal activity against *Pythium ultimum*, *Phytophthora infestans*, and *Ustilago maylis* (Halama and Van Haluwin, 2004). *Parmelina*

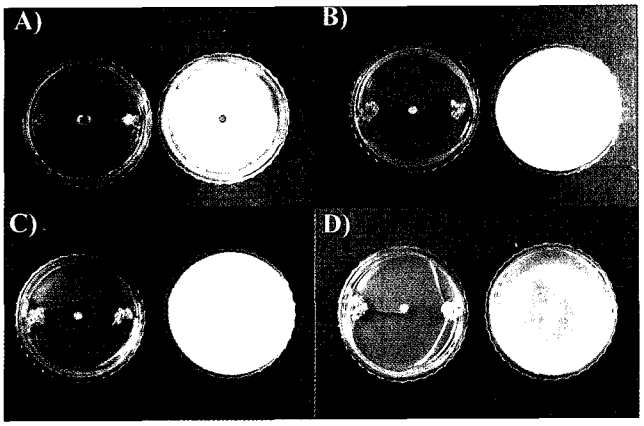

Fig. 2. Inhibition of mycelial growth of plant pathogenic fungi by LFF of *P. leavior*. Two mycelial masses of the isolate were placed at the edge of the plate 60 days before inoculation of fungal mycelial block of pathogenic fungi at the center of the plate. The inhibition rate was measured 3 to 5 days after inoculation. Mycelial growth of A) *Botryosphaeria dothidea*, B) *Pythium* sp., C) *Rhizoctonia solani* and D) *Sclerotium cepivorum* were completely inhibited by LFF of *P. laevior* (left), compared with normal growth of control (right).

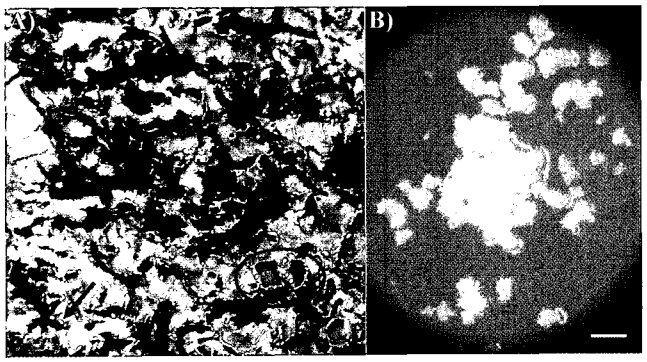

Fig. 3. *Parmelia laevior* (040257 HUR, Mt. Jiri). A) Lichen thalli having puctate marginal pseudocyphellae (allows) and apothecia (circle), B) LFF originated from discharged ascospores. (Bar = 1mm) after 1 month incubation.

quercina containing atranorin and salazinic acid as main lichen substance was also proved to have no antimicrobial activity (Wei et al., 1982). It is well known that LFF in axenic cultures retain the capacity to biosynthesize secondary products found in the lichenized state (Leuckert et al., 1990; Culberson et al., 1992), but the metabolites produced in the greatest abundance might differ from those found in the lichen (Miyagawa et al., 1993; Hamada, 1993). Therefore, it will be very interesting to investigate the compounds responsible for strong antifungal activity of the LFF in cultures. Mass cultivation of the LFF is now under progress in laboratory conditions for chemical identification of antifungal substances. In conclusion, the secondary metabolites of LFF in cultures might be of potential use as antifungal agents and LFF can serve as a novel bioresource to develop new biofungicides alternative to current fungicides to control plant pathogenic fungi.

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