Plant Pathol. J. 24(2) : 202-206 (2008)

The Plant Pathology Journal

Note

Antifungal Activity of Lichen-forming Fungi against *Colletotrichum acutatum* on Hot Pepper

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(Received on December 1, 2007; Accepted on March 26, 2008)

Antifungal activity of Korean and Chinese lichen-forming fungi (LFF) was evaluated against plant pathogenic fungus of *Colletotrichum acutatum*, causal agent of anthracnose on hot pepper. This is the first attempt to evaluate antifungal activity of LFF, instead of lichen thalli, against *C. acutatum*. Total 100 LFF were isolated from the lichens with discharged spore method or tissue culture method. Among the 100 isolates, 8 LFF showed more than 50% of inhibition rates of mycelial growth of the target pathogen. Especially, *Lecanora argentata* was highly effective in inhibition of mycelial growth of *C. accutatum* at the rate of 68%. Antifungal activity of other LFF was in the order of *Cetrelia japonica* (61.4%), *Ramalina conduplicans* (59.5%), *Umbilicaria esculenta* (59.5%), *Ramalina litoralis* (56.7%), *Cetrelia braunsiana* (56.5%), *Nephromopsis pallescensn* (56.1%), and *Parmelia simplicior* (53.8%). Among the tested LFF, 61 isolates of LFF exhibited moderate antifungal activity against the target pathogen at the inhibition rates from 30 to 50%. Antifungal activity of the LFF against *C. acutatum* was variable at the species level rather than genus level of LFF. This study suggests that LFF can be served as a promising bioresource to develop novel biofungicides.

Keywords **:** biofungicide, fungal growth inhibition, lichen-forming fungi, novel bioresource, plant pathogenic fungus

Pepper anthraconose caused by *Collectotrichum acutatum*, is one of the most important diseases in pepper cultivation in Korea (Choi et al., 2008; Kang et al., 2005). The disease was reported to be responsible for 10% annual yield loss of total pepper production in Korea, equivalent to approximately $0.1 billion per year (Shin et al., 1999). Current practice for controlling pepper anthracnose is mainly based on use of synthetic pesticides. However, many synthetic pesticides may lose their usefulness due to negative consequences for human health and the environment and development of resistance in pathogen populations (Russell, 1995). Recently, biological control has been developed as an alternative to synthetic pesticide treatment. A variety of microbial antagonists and their metabolites have been reported to control several different pathogens on various fruit and vegetables (Fravel, 2005).

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. Antifungal activity of lichen extracts and lichen acids against plant pathogenic fungi was reported (Gulluce et al., 2006; Halama and Van Haluwin, 2004; Oh et al., 2006). Nevertheless, the potential fungal obligate symbionts in lichen have long been neglected by mycologists and overlooked by agrochemical industry because of its slow growth in nature and difficulties in their artificial cultivation. The large-scale industrial production of the lichen metabolites has never been accomplished. However, use of lichen-forming fungi 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. This is the first attempt to screen antifungal activity against *C. acutatum* with use of large number of lichen-forming fungi, instead of natural lichen thallus, for further development of industrial production of natural fungicides.

The natural thalli of Korean lichens were collected from 20 mountain areas in South Korea during 2002 to 2004 (Hur et al., 2004). Chinese lichens were also collected from highland areas of Yunnan Province, China (Hur et al., 2005). Lichen specimens were air-dried for 1 week at room temperature and stored –20°C until isolation of lichen-forming fungi (LFF: mycobints). All the lichen materials were deposited at the herbarium of the Korean Lichen Research Institute, Sunchon National Univeristy, Korea. *Collectotrichum acutatum* KACC40042 was obtained from Korean Agricultural Culture Collection (KACC), Rural Development Administration (RDA), Suwon, Korea.

The colonies of LFF were obtained using discharged

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spore method (S in Table 1) and/or tissue culture method (T in Table 1) (Yoshimura et al., 2002). Details of LFF isolation using discharged spore method were previously described (Oh et al., 2006).

The fungal components of sterile lichens, or those in which isolation from discharged spore had not proved successful, were isolated from thallus fragments by the method of Yamamoto (Yoshimura et al., 2002). Small pieces of thallus such as 1 cm in length of fruticose lichen or 1 cm^2 of foliose and crustose lichen were cut from apical regions, washed in a turbulent flow of tap water for 1 h, then in excess sterile water, and finally macerated in a further 3-5 ml sterile water using a mortar and pestle. The resultant suspension was sieved through 500 μm and 150 μm nylon mesh filters in sequence with sterile water. Macerate retained on the 150 μm sieve was examined under a dissecting microscope and the fragments free from algae were picked out and transferred to agar media using sterilized stainless steel syringe-needle and inoculated onto the medium of 24 multi well (d=2 cm) plates. Total 94 inoculums from each lichen material were prepared. Cultures were incubated at 18°C in the dark and examined periodically during a week period. Thallus fragments that remained free of contamination were transferred to fresh medium. After 2-3 month's growth, mycobionts produced a compact mycelium 2-5 mm in diameter. These were subcultured onto fresh medium for fungal mass production. Species that continued to grow in subculture were recorded as successfully isolated. The culture medium of mal-extract agar was routinely used for isolation and growth of LFF (Yoshimura et al., 2002).

Analysis of the ribosomal DNA sequence of ITS region was attempted for molecular confirmation of the isolates obtained by tissue culture method. The sequences of resultant fungal mass of isolates and the original lichen thallus used for isolation were analyzed and compared. Fresh lichen thalli and fungal mass were fractioned with cryo-tissue-crasher (SK200, Tokken, Japan). Total DNA was extracted directly from whole thalli according to Ekman (1999) with DNeasy Plant Mini Kit (QIAGEN, Germany). 10^{-1} dilution of the total DNA was used for PCR amplification of the nuclear rDNA ITS and 5.8S genes. Primers for amplification were: ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White et al., 1990) and ITS5 (5'-GGAAGTAAAAGTCGRAACAAGG-3'; White et al., 1990). Conditions for PCR amplification and cycle sequencing have been described previously (Arup, 2002). PCR products were purified by PCRquick-spin™ PCR Product Purification Kit (iNtRON Biotechnology, INC.) and then sequenced using ABI 3700 automated DNA Sequencer in NICEM at Seoul National University.

Freshly grown two mycelial masses (3 mm diam.) of the isolated LFF were placed at the edge of malt extract agar 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 60 days before inoculation of *C. acutatum*. Freshly grown mycelium agar block (3 mm diam.) of *C. acutatum* was placed on the center of the pre-incubated agar plate. The inhibition zone of mycelial growth of the pathogenic fungus was rated 3 to 5 days after incubation at 18°C and compared with the control plate. Five replicate plates were used for the bioassay. Antifungal activity of the lichen-forming fungi was compared using one-way analysis of variance (ANOVA).

Total 100 lichen-forming fungal isolates were obtained from Korean and Chinese lichens using discharged spore method and tissue culture method (Table 1). Many ascospores were successfully discharged from a single apothecium and generally germinated within 1 week. Several fungal isolates was grown to visible size within 2 months. Lichen-forming fungi were also induced from lichen thalli by tissue culture method. Confirmation of lichen-forming fungi was carried out by ITS sequences analysis. Isolation rates of lichen-forming fungi from ascospore or lichen thalli were approximately 30% in this study (data not shown).

Some isolates leached large amount of pigments into the agar medium during the incubation. This suggested that secondary metabolites of LFF were produced and diffused into the medium. Compared with normal fungi, LFF grew 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).

Several LFF showed very strong antifungal activity against the pepper anthracnose pathogenic fungus (Table 1). Among the 100 isolates, 8 LFF showed more than 50% of inhibition rates of mycelial growth of the target pathogen. Especially, LFF of *Lecanora argentata* was mostly effective in inhibition of mycelial growth of *C. accutatum* at the rate of 68%. Antifungal activity of other LFF was in the order of *Cetrelia japonica* (61.4%) > *Ramalina conduplicans* (59.5 %) = *Umbilicaria esculenta* (59.5%) > *Ramalina litoralis* (56.7%) ≥ *Cetrelia braunsiana* (56.5%) ≥ *Nephromopsis pallescensn* (56.1%) > *Parmelia simplicior* (53.8%). Their antifungal activity was significantly higher than other lichen-forming fungi tested in this study ($P < 0.05$). Among the tested LFF, 61 isolates of LFF exhibited moderate antifungal activity against the target pathogen at the inhibition rates from 30 to 50%. Among the rest of 31 LFF isolates, only one LFF of *Cladonia cervicornis* showed less than 20% inhibition rate (17.8%) and others demonstrated recognizable antifungal activity from 20 to 30%. Antifungal activity of the LFF against *C. acutatum* was variable at the species level rather than genus level of LFF. How-

Table 1. Antifungal activity of various lichen-forming fungi isolated from Korean and Chinese lichens against hot pepper anthracnose pathogen, *Colletortichum acutatum in vitro*

Lichen species	Collection number	Locality	Isolation method[a]	Mycelium growth inhibition	
				Diam. (mm)[b]	% of control
Amandinea punctata (Hoffm.) Coppins & Scheid.	50623	Korea	T	36.54 ± 1.58	60.9
Anaptychia palmatula (Michx.) Vain.	41078	Korea	S	34.37 ± 0.11	57.3
Anaptychia palmatula (Michx.) Vain.	40180	Korea	T	34.36 ± 0.86	57.3
Anzia opuntiella Müll. Arg.	40280	Korea	S	43.97 ± 1.63	73.3
Bacidia schweinitzii (Tuck.) A. Schneid.	40162	Korea	S	38.94 ± 1.75	64.9
Bryoria confusa (D.D. Awasthi) Brodo & D. Hawksw.	CH050187	China	S	44.18 ± 0.76	73.6
Bryoria himalayensis (Motyka) Brodo & D. Hawksw.	CH050365	China	S	45.43 ± 3.54	57.7
Caloplaca flavorubescens (Huds.) J.R. Laundon	50696	Korea	T	40.76 ± 1.58	67.9
Cetrelia braunsiana (Müll. Arg.) W.L. Culb. & C.F. Culb.	40188	Korea	T	**26.67 ± 0.58**	**44.5**
Cetrelia japonica (Zahlbr.) W.L. Culb. & C.F. Culb.	30397	Korea	T	**23.18 ± 1.61**	**38.6**
Cetrelia braunsiana (Müll. Arg.) W.L. Culb. & C.F. Culb.	40425	Korea	T	40.48 ± 1.05	67.5
Cladonia furcata (Huds.) Schrad.	40034	Korea	T	45.96 ± 0.90	76.6
Cladonia gracilis subsp. *turbinata* (Ach.) Ahti	41582	Korea	S	42.43 ± 3.50	70.7
Cladonia gracilis (L.) Willd.	41474	Korea	S	35.46 ± 0.59	59.1
Cladonia metacorallifera Asahina.	41466	Korea	S	40.79 ± 2.45	68
Cladonia coniocraea (Flörke) Spreng.	040634-1	Korea	T	42.88 ± 1.37	71.5
Cladonia yunnana (Vain.) Abbayes.	CH050136	China	S	36.48 ± 1.10	60.8
Cladonia macilenta Hoffm.	CH050214	China	S	45.35 ± 2.47	75.6
Cladonia coccifera (L.) Willd.	CH050255	China	S	43.20 ± 1.71	72
Cladonia cervicornis (Ach.) Flot.	CH050299	China	T	49.31 ± 1.14	82.2
Cladonia pleurota (Flörke) Schaer.	CH050056	China	T	47.49 ± 3.83	79.2
Cladonia squamosissima (Müll. Arg.) Ahti	CH050180	China	T	43.84 ± 1.02	73.1
Cladonia rangiferina (L.) Weber ex F.H. Wigg.	CH050184	China	T	40.26 ± 1.00	67.1
Cladonia scabriuscula (Delise) Leight.	40481	Korea	S	46.41 ± 1.68	77.4
Everniastrum cirrhatum (Fr.) Hale ex Sipman.	CH050065	China	S	45.96 ± 1.44	76.6
Gymnoderma coccocarpum Nyl.	CH050374	China	T	39.98 ± 1.66	66.6
Heterodermia japonica (M. Satô) Swinscow & Krog.	CH050263	China	T	43.89 ±2.93	73.2
Heterodermia hypoleuca (Mühl.) Trevis.	40067	Korea	T	43.07 ± 3.55	71.8
Heterodermia hypoleuca (Mühl.) Trevis.	40598	Korea	T	40.99 ± 0.81	68.3
Heterodermia microphylla (Kurok.) Skorepa	40196	Korea	T	41.46 ± 1.87	69.1
Hypogymnia delavayi (Hue) Rass.	41338	Korea	S	37.7 ± 0.36	62.8
Hypogymnia pseudoenteromorpha M.J. Lai.	CH050143	China	S	41.65 ± 1.51	69.4
Hypogymnia pruinosa J.C. Wei & Y.M. Jiang.	CH050101	China	S	38.24 ± 1.22	63.7
Hypogymnia hengduanensis J.C. Wei.	CH050100	China	T	44.35 ± 1.06	73.9
Hypotrachyna osseoalba (Vain.) Y.S. Park & Hale	CH050261	China	T	44.76 ± 1.90	74.6
Icmadophila ericetorum (L.) Zahlbr.	CH050403	China	S	37.26 ± 3.31	62.1
Lecanora argentata (Ach.) Malme	50657	Korea	S	**19.02 ± 0.12**	**31.7**
Megalospora tuberculosa (Fée) Sipman.	50724	Korea	S	41.03 ± 3.00	68.4
Melanelia olivacea (L.) Essl.	40371	Korea	S	38.59 ± 1.11	64.3
Menegazzia terebrata (Hoffm.) A. Massal.	41300	Korea	T	44.24 ± 0.80	73.7
Menegazzia pseudocyphellata Aptroot, M.J. Lai & Sparrius.	CH050262	China	S	43.99 ± 0.17	73.3
Myelochroa aurulenta (Tuck.) Elix & Hale.	40664	Korea	T	39.13 ± 0.59	65.2
Myelochroa irrugans (Nyl.) Elix & Hale.	40974	Korea	T	32.85 ± 2.05	54.8
Myelochroa irrugans (Nyl.) Elix & Hale.	40303	Korea	T	33.82 ± 0.79	56.4
Myelochroa irrugans (Nyl.) Elix & Hale.	30730	Korea	T	33.53 ± 1.84	55.9
Myelochroa galbina (Ach.) Elix & Hale.	40954	Korea	S	41.93 ± 0.29	69.9
Myelochroa aurulenta (Tuck.) Elix & Hale.	40678	Korea	S	40.03 ± 2.15	66.7
Myelochroa indica (Hale) Elix & Hale.	40002	Korea	T	39.74 ± 1.97	66.2
Myelochroa entotheiochroa (Hue) Elix & Hale.	41602	Korea	S	33.27 ± 2.03	55.5
Nephromopsis yunnanensis (Nyl.) Randlane & Saag.	CH050123	China	S	35.16 ± 1.61	58.6
Nephromopsis pallescens (Schaer.) Y.S. Park.	CH050089	China	S	**26.96 ± 1.93**	**44.9**
Nephromopsis ornate (Müll. Arg.) Hue.	41359	Korea	T	38.21 ± 2.20	63.7

Table 1. Continued

Lichen species	Collection number	Locality	Isolation method[a]	Mycelium growth inhibition	
				Diam. (mm)[b]	% of control
Nephromopsis asahinae (M. Satô) Räsänen.	40500	Korea	S	40.64 ± 2.15	67.7
Nephromopsis pseudocomplicata (Asahina) M.J. Lai	CH040025	China	S	37.34 ± 1.15	62.2
Parmelia simplicior Hale	CH050260	China	S	**27.69 ± 4.02**	**46.2**
Parmelia laevior Nyl.	40257	Korea	S	41.46 ± 0.29	69.1
Parmelia adaugescens Nyl.	40810	Korea	S	32.65 ± 2.38	54.4
Parmelia pseudolaevior Asahina.	50069	Korea	S	34.71 ± 2.64	57.9
Parmelia omphalodes (L.) Ach.	40356	Korea	T	38.11 ± 2.37	63.5
Parmotrema austrosinense (Zahlbr.) Hale.	CH050252	China	T	40.83 ± 2.79	68.1
Parmotrema ultralucens (Krog) Hale.	CH050249	China	T	40.83 ± 2.45	68.1
Pannaria leucosticta Tuck.	41227	Korea	T	32.41 ± 0.54	54
Phaeophyscia limbata (Poelt) Kashiw.	40014	Korea	S	43.20 ± 2.06	72
Phaeophyscia exornatula (Zahlbr.) Kashiw.	40202	Korea	T	43.77 ± 1.35	73
Phaeophyscia melanchra (Hue) Hale.	40625	Korea	S	41.87 ± 2.06	69.8
Physcia caesia (Hoffm.) Fürnr.	40912	Korea	S	39.41 ± 1.05	65.7
Phaeophyscia exornatula (Zahlbr.) Kashiw.	40923	Korea	T	43.73 ± 4.76	72.9
Phaeophyscia hirtella Essl.	41166	Korea	S	47.47 ± 4.07	79.1
Physcia stellaris (L.) Nyl.	41642	Korea	S	42.88 ± 2.06	71.5
Physcia stellaris (L.) Nyl.	50213	Korea	S	44.69 ± 0.92	74.5
Phaeophyscia melanchra (Hue) Hale.	40089	Korea	T	40.74 ± 0.16	67.9
Punctelia borreri (Sm.) Krog	40051	Korea	T	39.00 ± 1.78	65
Pyxine endochrysina Nyl.	41658	Korea	T	34.21 ± 0.40	57
Pyxine consocians Vain.	40935	Korea	S	30.77 ± 1.07	51.3
Ramalina exilis Asahina.	30474	Korea	T	45.67 ± 0.81	76.1
Ramalina intermedia Delise ex Nyl.	CH050064	China	S	37.45 ± 0.16	62.4
Ramalina pertusa Kashiw.	40628	Korea	T	38.90 ± 1.50	64.8
Ramalina litoralis Zahlbr.	50319	Korea	S	**26.58 ± 1.31**	**44.3**
Ramalina conduplicans Vain.	40402	Korea	S	**24.28 ± 2.41**	**40.5**
Ramalina sp.	CH040302	China	S	39.15 ± 2.49	65.3
Ramalina complanata Ach.	CH050099	China	S	29.89 ± 0.92	49.8
Ramalina yasudae Räsänen.	30330	Korea	T	35.69 ± 1.41	59.5
Ramalina almquistii Vain.	30237	Korea	S	40.92 ± 0.62	68.2
Ramalina sinensis Jatta.	CH040020	China	S	45.94 ± 0.26	76.6
Ramalina roesleri (Hochst. ex Schaer.) Hue.	CH050380	China	T	42.62 ± 1.33	71
Rimelia clavulifera (Räsänen) Kurok.	50038	Korea	S	36.80 ± 2.38	61.3
Rimelia clavulifera (Räsänen) Kurok.	50150	Korea	S	41.95 ± 1.78	69.9
Rimelia reticulata (Taylor) Hale & A. Fletcher.	CH050393	China	T	42.61 ± 1.87	71
Rimelia reticulata (Taylor) Hale & A. Fletcher.	40068	Korea	T	39.68 ± 3.04	66.1
Stereocaulon commixtum (Asahina) Asahina.	40659	Korea	S	44.63 ± 1.92	74.4
Tephromela atra (Huds.) Hafellner.	40191	Korea	S	42.84 ± 3.05	71.4
Tuckneraria pseudocomplicata (Asahina) Randlane & Saag	40516	Korea	S	41.73 ± 3.15	69.6
Umbilicaria proboscidea (L.) Schrad.	CH040077	China	S	39.65 ± 0.72	66.1
Umbilicaria kisovana (Zahlbr. ex M. Satô) Kurok.	30221	Korea	T	37.52 ± 0.28	62.5
Umbilicaria esculenta (Miyoshi) Minks.	40040	Korea	T	**24.31 ± 1.12**	**40.5**
Umbilicaria yunnana (Nyl.) Hue.	CH050097	China	T	40.36 ± 2.10	67.3
Usnea longissima Ach.	CH050148	China	S	42.25 ± 0.65	70.4
Usnea orientalis Motyka.	CH050316	China	S	29.94 ± 1.68	49.9
Xanthoparmelia hirosakiensis (Gyeln.) Kurok.	50141	Korea	S	38.42 ± 1.27	64
Xanthoria elegans (Link) Th. Fr.	41247	Korea	S	34.31 ± 0.58	57.2
Control				60.00	–

[a]S; Discharged spore method, T; Tissue culture method.

[b]The inhibition zone of mycelial growth of the pathogenic fungus was rated 3 to 5 days after incubation at 18°C and compared with the control plate. Data represent the mean and standard deviation of five replications. Bold letters indicate the lichen-forming fungi which showed significantly higher antifungal activity against *C. acutatum* than other lichen-forming fungi tested in this study ($P < 0.05$, LSD) .

ever, LFF isolated from lichen species belong to Cetrariod genera (*Cetrelia* and *Nephromposis*) and *Ramalina* genus possessed stronger antifungal activity against the target pathogenic fungus than those isolated from other lichen genera tested in this study. Mycelial growth of *Botryosphaeria dothidea*, *Botrytis cinerea*, *Pythium* sp., *Rhizoctonia solani* and *Sclerotium cepivorum* were completely inhibited by LFF of *Parmelia laevior* in our previous study (Oh et al., 2006). However, it was not the case for *C. acutatum* in this study. This suggests that secondary metabolites produced during LFF culture have differential sensitivity to various plant pathogenic fungi and the compounds acted in a species-specific manner.

Methanol or acetone extracts of several lichen thalli were already proved to have strong antifungal activity against various plant pathogenic fungi (Gulluce et al., 2006; Halama and Van Haluwin, 2004). Unlike the previous results using intact lichen thalli extracts, LFF exhibited different antifungal activity against *Colletotrichum* fungus in this study. It is well known that LFF in axenic cultures retain the capacity to biosynthesize secondary products found in the lichenized state (Culberson et al., 1992), but the metabolites produced in the greatest abundance might differ from those found in the lichen (Miyagawa et al., 1993). Therefore, it will be very interesting to investigate the compounds responsible for strong antifungal activity of the LFF in cultures with comparison of natural lichen substances. 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 bioresources to develop new biofungicides alternative to current fungicides to control *C. acutatum*, hot pepper anthracnose pathogenic fungus.

Acknowledgements

This work was supported in part by Rural Development Administration program (Grant 20070301-033-016-001-02-00), Republic of Korea and by NON DIRECTED RESEARCH FUND (2004), Sunchon National University, Korea.

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