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Isolation of Lichen-forming Fungi from Hungarian Lichens and Their Antifungal Activity Against Fungal Pathogens of Hot Pepper Anthracnose

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Lichen-forming fungi (LEF) were isolated from 67 Hungarian lichen species from ascospores or thallus fragments. LFF were successfully isolated from 26 species with isolation rate of 38.8%. Of the total number of isolation from ascospores (27 species) and thallus fragments (40 species), 48% and 32.5% of the species were successfully isolated, respectively. Comparison of rDNA sequences of ITS regions between the isolated LFF and the original thallus confirmed that all the isolates originated from the thallus fragments were LEF. The following 14 species of LEF were newly isolated in this study; *Acarospora cervina, Bacidia rubella, Cladonia pyxidata, Lasallia pustulata, Lecania hyaline, Lecanora argentata, Parmelina tiliacea, Parmotrema chinense, Physconia distorta, Protoparmeliopsis muralis, Ramalina pollinaria, Sarcogyne regularis, Umbilicaria hirsuta, Xanthoparmelia conspersa* and *X. stenophylla*. Antifungal activity of the Hungarian LFF was evaluated against plant pathogenic fungi of *Colletotrichum acutatum, C. coccodes* and *C. gloeosporioides*, causal agent of anthracnose on hot pepper. Among the 26 isolates, 11 LFF showed more than 50% of inhibition rates of mycelial growth of at least one target pathogen. Especially, LFF of *Evernia prunastri, Lecania hyalina* and *Lecanora argentata* were remarkably effective in inhibition of mycelial growth of all the tested pathogens with antibiotic mode of action. On the other hands, five isolates of *Cladonia furcata, Hypogymnia physodes, Lasallia pustulata, Ramalina fastigiata* and *Ramalina pollinaria* exhibited fungal lytic activity against all the three pathogens. Among the tested fungal pathogens, *C. coccodes* seemed to be most sensitive to the LFF. The Hungarian LFF firstly isolated in this study can be served as novel bioresources to develop new biofungicides alternative to current fungicides to control hot pepper anthracnose pathogenic fungi.

Keywords : Antifungal activity, biofungicide, bioresource, lichen-forming fungi, hot pepper anthracnose

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Lichen is a stable, self-supporting, and mutualistic symbiont composing of a fungus (the mycobiont) and a green alga and/or a cyanobacterium (the photobiont). The 13,500 species of lichen-forming fungi described constitute about 42% of all known Ascomycota, about one fifth of all known fungi (Kirk et al., 2001) and therefore, a major group of fungal kingdom. Lichens produce characteristic secondary metabolites, which seldom occur in other organisms. Lichens and their metabolites have many biological activities such as antimicrobial, antifungal, antiviral, antiprotozoal, antiproliferative, antioxidant and anti-inflammatory (Behera et al., 2005; Halama and Van Halywin, 2004; Ingolfsdottir, 2002; Müller, 2001; Perry et al., 1999; Yamamoto et al., 1998). Antifungal activity of lichen extracts and lichen acids against plant pathogenic fungi were also reported (Gulluce et al., 2006; Halama and Van Haluwyn, 2004; Wei et al., 2008). In spite of the wide spectrum of biological activities shown by the lichens, they have long been neglected by mycologists and overlooked by pharmaceutical and agrochemical industry because of its slow growth in nature and difficulties in the artificial cultivation of organisms (Crittenden and Porter, 1991). Hence 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.

During the last two decades, studies on lichen-forming fungi in pure culture have accelerated in university laboratories in the USA, Sweden, Germany and Japan, primarily for the purposes of taxonomic and physiological studies. Recently, isolation and culture of unexploited lichen-forming fungi have been launched in Asian countries such as Korea, India and Thailand mainly for biological activities of lichen secondary metabolites (Behera et al., 2006a, 2006b; Wei et al., 2008). The Korean Lichen Research Institute (KoLRI) opened Lichen and Allied Bioresource Bank which plays a key role in distributing lichen bioresources to research institutes, industrial sectors and university laboratories in

Korea. In a systemic scheme of the bioresource collection, oversea lichen collection and isolation of lichen-forming fungi from them have been attempted for the last few years in KoLRI.

Pepper anthracnose is one of the most severe diseases in pepper cultivation in Korea (Kang et al., 2005). The anthracnose is rapidly spread by fungal spores in rainy season and often results in whole crop loss. The disease was reported to be account for 10% annual yield loss of total pepper production in Korea, equivalent to approximately 0.1 billion USD per year (Shin et al., 1999). Current practice for controlling pepper anthracnose is mainly based on use of synthetic pesticides in Korea. However, many of the synthetic pesticides already lost their usefulness due to development of resistance in pathogen populations. The emergency of fungicide-resistant strains of *C. acutatum* is currently considered to be the main barrier for the control of pepper anthracnose in Korea. In addition, synthetic pesticides were known to have negative consequences for human health and the environment.

In this study, we attempted isolation of lichen-forming fungi from Hungarian lichens as a potential bioresource of agrochemical application. For this purpose, antifungal activity of the isolates was also screened against *C. acutatum*, *C. coccodes* and *C. gloeospoioides* to provide novel antifungal agents which can be alternatives to current fungicides for pepper anthracnose control.

Materials and Methods

Lichen collection and identification

One hundred thirty three specimens were collected at the following 3 locations on May 2006 (Fig. 1). The expedition of lichen collection was officially organized by The Hungarian Natural History Museum. These collections are deposited in the Korean Lichen Research Institute (KoLRI) at Sunchon National University of Korea.

Vértes Mts form the middle section of the Transdanubian Mountain Range. It is a hilly area with average height of 350 m, and its highest peak is just 480 m. The bedrock is calcareous (dolomite, Dachstein limestone, etc.). The exposed rock surfaces are covered mainly by grasslands. The forests and scrublands are composed of *Quercus pubescens*, *Fraxinus ornus*, *Cornus mas*, *Cotinus coggygria*, *Sorbus* spp., *Carpinus betulus*, *Fagus sylvatica*, etc. The vegetation of the southern slopes has sub-Mediterranean character. On the other hand, narrow gorges between the stone walls keep cooler climate. Among others, the most famous protected plant here is *Primula auricula*, which is regarded as a glacial relict plant from the cooler periods of the Ice Age. Another relict species is *Carpinus orientalis* a living remnant possibly from the warmer periods before the Ice Age. The Vértes Landscape Protection area established in 1976 on 150 km^2 now belongs to the Duna-Ipoly National Park.

Bükk Mts are situated in the centre of the Northern Mountain Range. Most of its territory belong to the Bükk National Park, which is also very famous of its geological monuments like cliffs, caves, etc. It received protection in 1977 and now it covers 433 km^2. Most of the area is above 500 m, the highest peak is 959 m. The central part is

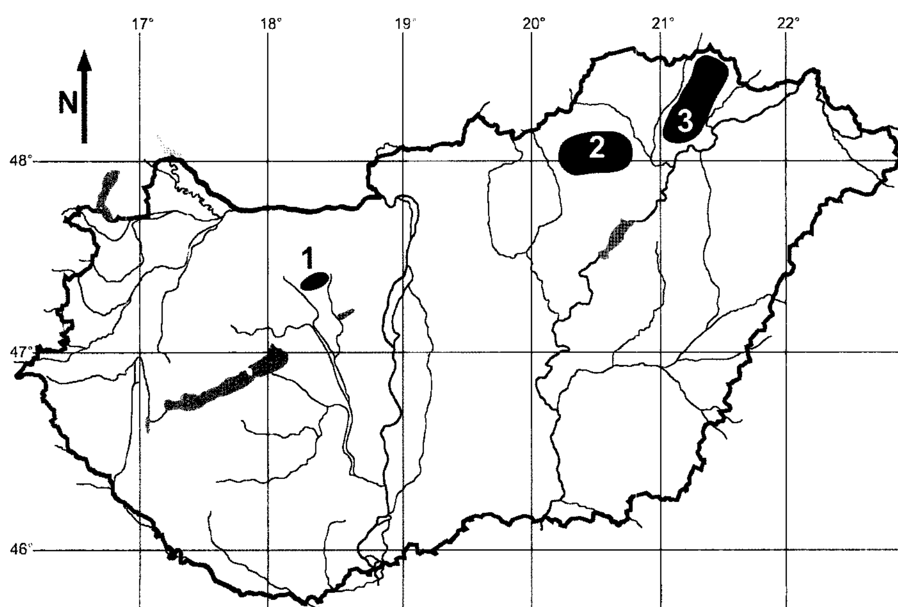

Fig. 1. Collecting localities in Hungary. 1=Vértes Mts (Nos H06003-H06029), 2=Bükk Mts (Nos H06031-H06046), 3= Zemplén Mts (Nos H06049-H06135).

occupied by a big karst plateau, called Bükk plateau. The bedrock is mainly calcareous (limestone, dolomite, etc.), but various acidic rock types (schist, radiolarite, rhyolite, etc.) also occur. The most remarkable karst formations are located on the Bükk plateau. Its climate is of montane character, moderately cool and humid. The vegetation shows a characteristic altitudinal zonation. At higher altitudes and on steep northern slopes *Fagus sylvatica* is dominated, mixed with some coniferous plantations in some places. Mixed hornbeam-oak woods, acidophilous or basiphilous oakwoods usually occur at the lower zones. Open exposed rock surfaces are covered by rocky grasslands. Due to the geographical conditions Bükk Mts have a rich and diverse flora (ca 1,500 species) with lots of montane species. The most famous glacial relicts here are *Dracocephalum ruyschiana* and *Viola biflora*. Endemic plants are also living here, e.g. *Ferula sadleriana*.

Zemplén Mts are the easternmost part of the Northern Mountain Range with a direct continuation toward the Carpathians. Its average height is ca 600 m, its highest peak is 896 m at the Hungarian-Slovak border. It has a volcanic origin, the main bedrock is of acidic character (andesite, dacite, rhyolite, perlite, etc.). Its climate is also cool and humid. Most of the area (85%) are covered by deciduous forests (beech, hornbeam, oak, etc.). Due to the diverse and various habitats (rocky habitat, water habitat, etc.) it has a unique and diverse flora rich in species (ca 1,350 species) including several rare Carpathian flora elements. The castle hill of Füzér is one of the most important site for rare and protected plants. The Zemplén Landscape Protection Area was founded in 1984 with the territory of 265 km^2. It is also part of the Bükk National Park.

Identification of lichens was made mainly by Dr. L. Lökös, (The Hungarian Natural History Museum, Budapest, Hungary). Where appropriate, the nomenclature follows IndexFungorum. Recent generic treatments are adopted for this study. Thin-layer chromatography (TLC) was also performed to identify lichen chemical compounds with three developing solvent systems (Culberson, 1972).

Isolation and confirmation of lichen-forming fungi (LFF) For the majority of lichens bearing reproductive structures (fruiting bodies), the LFF were obtained using discharged spore method (Yoshimura et al., 2002). Discharged spores were observed under the microscope and the germinated spores were transferred to fresh medium for the isolation (Fig. 2). After 2-3 month's incubation at 18°C in the dark, the germinated spores produced compact mycelia with 2-5 mm in diameter and then were subcultured onto fresh medium. Species that continued to grow in subculture were recorded as successful isolation. The culture medium of malt-extract agar was routinely used for isolation and growth of LFF.

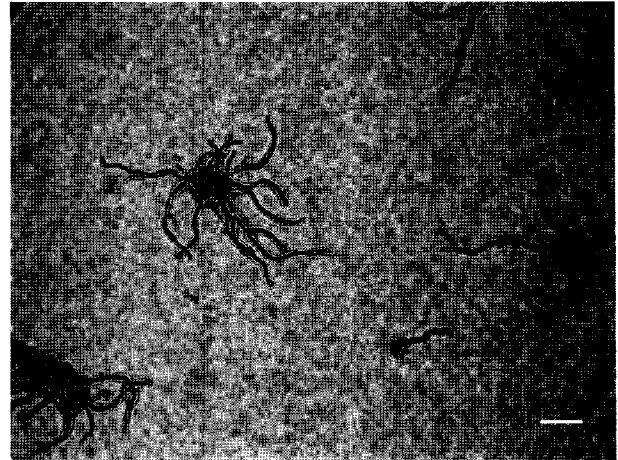

Fig. 2. Germination of discharged ascospores of *Lecanora argentata* (H060115-1) observed under invert microscope. Ascospore discharge, spore germination and growth of germinated spores were periodically examined using discharged spore method. Bar=20 mm.

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's (Yamamoto et al., 1985). Details of the isolations were previously described (Wei et al., 2008). 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. The resultant mycelial mats were used for the confirmation of LFF. Analysis of the ribosomal DNA sequence of ITS region was attempted for molecular confirmation of the isolates originated from the thallus fragments. The nucleotide sequences of the resultant fungal mass of the 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). Dilutions (10^{-1}) of the total DNA were 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.

Antifungal activity of the LFF isolates

Antifungal activity of the isolates was evaluated by dual plate assay. Freshly grown two mycelial masses (3 mm in diameter) 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 3 pathogenic fungi of *Collectotrichum acutatum*, *C. coccodes* and *C. gloeospoioides* causing pepper anthracnose. These pathogens were obtained from Korean Agricultural Culture Collection (KACC), Rural Development Administration (RDA), Suwon, Korea. Freshly grown mycelium agar block (6 mm diam.) of the fungal pathogens was placed on the center of the pre-incubated agar plate. The diameter of mycelial mats of the pathogenic fungi was measured 5 to 7 days after incubation at 18°C and compared with the control plate. Five replicate plates were used for the bioassay.

Results and Discussion

Isolation of lichen-forming fungi

Among 67 species tried, lichen-forming fungi (LFF) were successfully isolated from 26 species with isolation rate of 38.8% (Table 1). Of the total number of lichen species attempted from ascospores (27 species) and thallus fragments (40 species), 13 species (48%) and 13 species (32.5%) of the LFF isolates were successfully obtained, respectively. The isolation rates were high as almost 10% as those previous reported (Crittenden et al., 1995). This could be attributed to the substratum and collection period of our lichen materials. Harvesting period of apothecium in the field was found to be a critical factor for isolation of lichen-forming fungi from ascospores in temperate regions of Northern Hemisphere (Yamamoto, 2002). The highest rate (<90%) of discharged ascospores was recorded from the apothecia harvested in spring season (March to May). The lichen materials used in our study were collected in the middle of May. There were no significant seasonal variations in isolation of LFF from thallus fragment (Yamamoto et al., 2002). Collecting localities possibly affected the contamination and growth of thallus fragments and thus, lichen species growing on soil were highly contaminated. Higher contamination rates were also found in the lichen species having soredia and soralia than those having a smooth surface or containing antibiotic substances.

The reason for failure to isolate from ascospores can be as follows; no discharge of ascospores, failure of discharged ascospore germination, ascospores germinated but no growth. According to Ahmadjian (1993), ascospores of about 50% of a randomly collected group of lichens are likely to fail to germinate for unknown reasons. There was also possibility that the weather conditions at the collection could affect isolation rate of LFF from ascospore (Crittenden et al., 1995). They found that lichen materials collected on the same excursion but under different weather conditions responded differently; specimens gathered in wet weather tended to give better results than those collected during the same expedition but under arid conditions. Because of comparatively long-lived fruit bodies of lichens, fungal tissues of mycobiont heavily impregnate with materials that render the ascomata resistant to desiccation and decay. These features mean that the ascomata can remain long after ascospores have ceased to be produced; this could explain why failure to discharge ascospores was the reason for isolation failure in some lichen species (Crittenden et al., 1995). In the case of isolation failure with thallus fragments, most of them were due to failure of the fragments to grow and contamination of mostly yeast/bacteria and filamentous fungi was another reason for the failure.

Petrini et al. (1990) isolated and identified large numbers of filamentous fungi from thallus fragments of *Cladonia* and *Stereocaulon* spp., prepared by Yamamoto et al. (1985). This finding implied that at least some of the contaminants could occur within the lichen thallus and some of the isolates obtained from thallus fragments could be the contaminants. It is not easy to distinguish LEF from the contaminants among the isolates by cultural characteristics such as growth rate, morphology and color. To confirm LEF among the isolated fungal colony originated from thalllus fragments, we compared rDNA sequences of ITS regions from isolated colonies and the original thallus. LEF showed a high sequences homology to the original thallus with more than 98% identities (Table 1). This result suggests that molecular analysis of ITS regions could be a useful tool to confirm the isolated LEF among fungal colonies obtained from thallus fragments.

Isolation of LEF from Hungarian lichens was firstly attempted in this study. As a result, total of 26 species of LEF including 14 newly isolated species were obtained. Based on previous records of LEF available from literature (Crittenden et al., 1995; Yoshimura et al., 2002) and American and European internet sources (ATCC and CABRI), the following 14 species of LEF were newly isolated in this study; *Acarospora cervina, Bacidia rubella, Cladonia pyxidata, Lasallia pustulata, Lecania hyaline, Lecanora argentata, Parmelina tiliacea, Parmotrema chinense, Physconia distorta, Protoparmeliopsis muralis, Ramalina pollinaria, Sarcogyne regularis, Umbilicaria hirsuta, Xanthoparmelia conspersa* and *X. stenophylla*. All these isolates are ready to be distributed to all users for non-profit research purpose.

Antifungal activity of the isolates against pepper anthr-

Table 1. Lichen-forming fungi isolated from Hungarian lichens with use of ascospores or thallus fragments

Collection number	Lichen species	Isolation	Method[a]	Homology (%)	Accession number	Yamamoto (2002)	Crittenden (1995)	ATCC[b]	CABRI[c]
H060013	***Acarospora cervina***[d]	•	T	100					
H06064	*Acarospora fuscata*		T			•	•	•	
H060123	*Acrocordia gemmata*		S						
H060122	***Bacidia rubella***	•	S		EU266078				
H06012	*Caloplaca aurantia*		S						
H06016	*Caloplaca dolomiticola*		S						
H06029	*Caloplaca flavescens*		S						
H06094	*Candelariella coralliza*		T					•	
H06066	*Cetrelia olivetorum*		T			•	•		
H060111	*Chrysothrix chlorina*		T						
H06082	*Cladonia chlorophaea*		T			•	•		
H060049-1	*Cladonia coniocraea*	•	T	100			•		
H06049-2	*Cladonia fimbriata*		T						
H060059	*Cladonia furcata*	•	T	98	EU266080				
H060121	***Cladonia pyxidata***	•	T	99					
H06046	*Collema cristatum*		S						
H060110	*Collema flaccidum*		T						
H060109	*Dermatocarpon miniatum*		T				•		
H060097	*Diploschistes scruposus*	•	S			•			
H060020	*Evernia prunastri*	•	S		EU266079	•			
H060069	*Flavoparmelia caperata*		T			•			
H06087	*Hypocenomyce scalaris*		T			•			
H060125	*Hypogymnia physodes*	•	T	100		•			
H06085	*Imshaugia aleurites*		T						
H060099	***Lasallia pustulata***	•	T	99					
H06032	*Lecania erysibe*		S			•	•		
H060115	***Lecania hyalina***	•	S						
H060115-1	***Lecanora argentata***	•	S						
H060119-1	*Lecanora carpinea*		S						
H060038	*Lecanora dispersa*	•	S		EU266081	•	•	•	
H06070	*Lecanora conizaeoides*		S			•	•		
H060117	*Lecidella elaeochroma*	•	S		EU266082	•	•		
H06096	*Lepraria caesioalba*		T						
H06040	*Leptogium lichenoides*		T						
H060007	*Lobothallia radiosa*		T						
H060103	*Melanelia fuliginosa*	•	T	100			•		
H060100	*Neofuscelia pulla*	•	S		EU266083	•	•		
H060052	*Parmelia saxatilis*	•	T	100		•	•		
H0600131	*Parmelia sulcata*	•	S		EU266084	•	•		
H060022	***Parmelina tiliacea***	•	T	99	EU266085				
H060073	*Parmotrema chinense*		T						
H060120	*Peltigera horizontalis*		S						
H060116	*Peltigera praetextata*		T			•			
H06015	*Peltigera rufescens*		T						
H06080	*Pertusaria leioplaca*		S			•	•		
H06018-1	*Phaeophyscia nigricans*		T						
H06028	*Phaeophyscia orbicularis*		T						
H060102	*Phlyctis argena*		T						
H06025	*Physcia adscendens*		T			•	•		
H06006	*Physcia caesia*		T						
H06034	***Physconia distorta***	•	T	99	EU266086				
H06009	*Placocarpus schaereri*		T						
H06086	***Protoparmeliopsis muralis***	•	S		EU266090				

Table 1. Continued

Collection number	Lichen species	Isolation	Method[a]	Homology (%)	Accession number	Yamamoto (2002)	Crittenden (1995)	ATCC[b]	CABRI[c]
H06053	*Pseudevernia furfuracea*		T			•			
H06068	*Punctelia subrudecta*		T						
H060127	*Ramalina fastigiata*	•	T	100		•	•		
H060019	***Ramalina pollinaria***	•	T	99					
H06095	*Rhizocarpon geographicum*		S						
H060045	***Sarcogyne regularis***		S		EU266087				
H06005	*Squamarina cartilaginea*		S			•	•		
H06026	*Squamarina lentigera*		S						
H060091	***Umbilicaria hirsuta***	•	T	100	EU266088				
H06010	*Verrucaria nigrescens*		T						
H060135	***Xanthoparmelia conspersa***	•	S		EU266091	•	•		
H060083	***Xanthoparmelia stenophylla***	•	S		EU266089				
H06031	*Xanthoria fallax*		T						
H060114-1	*Xanthoria parietina*		S				•	•	

[a]S: discharged spore method, T: tissue culture method
[b]ATCC: http://www.atcc.org/
[c]CABRI (Common Access to Biological Resources and Information): http://www.cabri.org/
[d]Bold letters indicated the newly isolated LFF in this study.

Table 2. Antifungal activity of Hungarian lichen-forming fungi in dual plate assay

Collection number	Lichen species	Diameter (mm) of the mycelium[a]		
		C. acutatum	*C. coccodes*	*C. gloeosporioides*
H060013	*Acarospora cervina*	12.22 ± 0.78	**5.26 ± 1.43**	20.47 ± 1.33
H060122	*Bacidia rubella*	22.11 ± 2.23	23.15 ± 0.90	27.73 ± 0.38
H060049-1	*Cladonia coniocraea*	20.95 ± 0.54	**1.44 ± 0.34**	23.33 ± 0.11
H060059	*Cladonia furcata*	22.82 ± 2.26	14.00 ± 1.17	26.87 ± 2.31
H060121	*Cladonia pyxidata*	22.87 ± 1.05	33.91 ± 0.87	32.40 ± 1.32
H060097	*Diploschistes scruposus*	16.17 ± 1.97	35.67 ± 1.53	33.68 ± 0.57
H060020	*Evernia prunastri*	**6.23 ± 2.04**	**1.18 ± 0.08**	**7.52 ± 2.27**
H060125	*Hypogymnia physodes*	22.97 ± 0.43	20.19 ± 0.42	21.44 ± 0.66
H060099	*Lasallia pustulata*	27.14 ± 2.33	13.64 ± 0.49	25.62 ± 0.32
H060115	*Lecania hyalina*	**1.18 ± 0.16**	**1.23 ± 0.13**	**1.53 ± 0.81**
H060115-1	*Lecanora argentata*	**10.62 ± 1.37**	**1.39 ± 0.11**	**4.79 ± 1.53**
H060117	*Lecidella elaeochroma*	25.37 ± 2.44	21.15 ± 0.21	33.23 ± 0.45
H060103	*Melanelia fuliginosa*	20.95 ± 0.86	25.73 ± 1.02	22.75 ± 1.09
H060100	*Neofuscelia pulla*	22.34 ± 2.20	14.54 ± 1.12	27.43 ± 0.37
H060052	*Parmelia saxatilis*	11.14 ± 1.00	18.06 ± 0.94	29.16 ± 0.90
H0600131	*Parmelia sulcata*	12.14 ± 0.41	**7.29 ± 0.42**	19.14 ± 0.91
H060022	*Parmelina tiliacea*	24.40 ± 0.85	19.56 ± 1.18	30.16 ± 0.52
H06034	*Physconia distorta*	23.92 ± 0.95	35.22 ± 0.90	35.57 ± 0.66
H06086	*Protoparmeliopsis muralis*	19.37 ± 1.34	24.66 ± 1.15	26.40 ± 1.15
H060127	*Ramalina fastigiata*	30.27 ± 1.19	26.43 ± 0.91	34.67 ± 0.55
H060019	*Ramalina pollinaria*	25.33 ± 0.58	31.32 ± 0.92	33.06 ± 1.10
H060045	*Sarcogyne regularis*	21.81 ± 0.70	22.41 ± 1.01	30.16 ± 1.44
H060091	*Umbilicaria hirsuta*	19.85 ± 0.54	23.93 ± 0.20	29.71 ± 1.74
H060135	*Xanthoparmelia conspersa*	22.07 ± 0.10	14.99 ± 1.27	26.02 ± 0.95
H060083	*Xanthoparmelia stenophylla*	19.87 ± 0.81	18.43 ± 1.60	21.97 ± 3.15
	Control	21.95 ± 1.17	36.81 ± 0.28	36.27 ± 0.83

[a]Data represent the means and standard deviations of five replicates. The diameter of mycelial mats was measured 5 to 7 days after inoculation of the pathogenic fungi. Bold letters indicate the lichen-forming fungi which showed significantly higher antifungal activity.

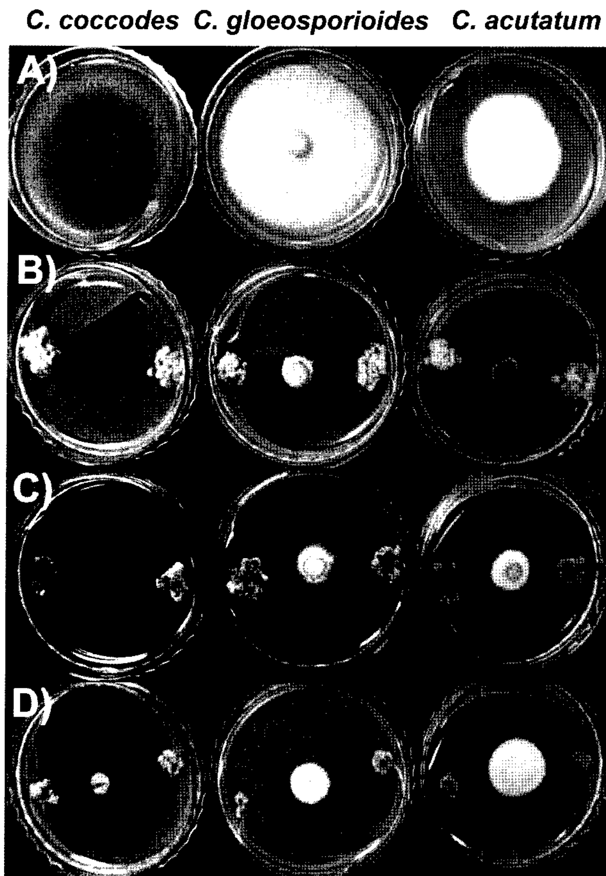

Fig. 3. Antifungal activity of Hungarian LFF with antibiotic mode of action in dual plate assay. A) Control, B) *Evernia prunastri*, C) *Lecania hyaline* and D) *Lecanora argentata*. The LFF were incubated on MY medium at 18°C in dark condition 60 days before inoculation of the pathogenic fungi. The diameter of mycelial mats of the pathogenic fungi was measured 5 to 7 days after inoculation.

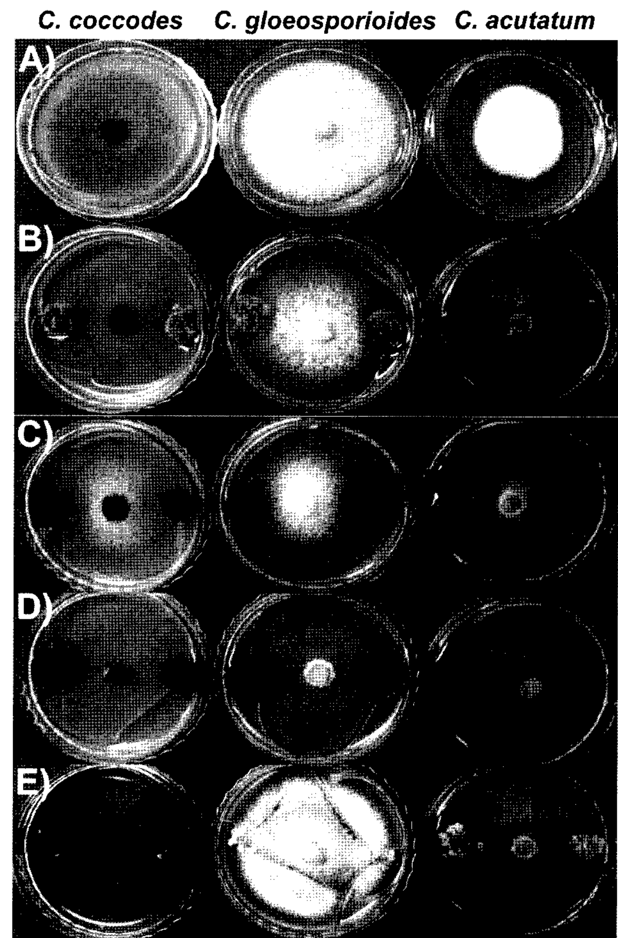

Fig. 4. Antifungal activity of Hungarian LFF with fungal lytic mode of action in dual plate assay. A) Control, B) *Cladonia furcata*, C) *Hypogymnia physodes*, D) *Lasallia pustulata* and E) *Ramalina fastigiata*. The LFF were incubated on MY medium at 18°C in dark condition 60 days before inoculation of the pathogenic fungi.

acnose fungal pathogens. Some LFF showed strong antifungal activity against the pepper anthracnose pathogenic fungi (Table 2). Among the 26 isolates, 11 LFF showed more than 50% of inhibition rates of mycelial growth of at least one target pathogen. Especially, LFF of *Evernia prunastri*, *Lecania hyalina* and *Lecanora argentata* were remarkably effective in inhibition of mycelial growth of all the tested pathogens (Fig. 3). For example, *L. hyaline* exhibited the inhibition at the rates of 94.6%, 96.6% and 95.8% against *C. acutatum*, *C. coccodes* and *C. gloeosporioides*, respectively, compared to controls. Unlike fungicidic or fungistatic activity, fungal lytic activity was also detected in dual plate assay. Among 26 LFF, five isolates *of Cladonia furcata*, *Hypogymnia physodes*, *Lasallia pustulata*, *Ramalina fastigiata* and *Ramalina pollinaria* exhibited fungal lytic activity against all the three pathogens (Fig. 4). The pathogenic fungi showed normal growth of mycelia at the early dual culture, but fungal hyphae of the pathogenic fungi became disentangled and pulpy in a lytic pattern at the late incubation. On the other hand, there was no lytic degradation of fungal hyphae on control plate where the pathogenic fungus was inoculated alone. This observation suggested that the lichen-forming fungi might produce and excrete the enzymes responsible for cell wall degradation of the pathogenic fungi. Lichens contain a rich diversity of cell wall enzymes, including phosphatases, cellulases, ureases, and also several redox enzymes such as laccases, tyrosinases, and peroxidases (Beckett and Minibayeva, 2007; Laufer et al., 2006). β-1.4-glucanase was also reported in soil lichens such as Peltigera (de los Rýós et al., 1997).

Differential sensitivity of the pathogens to the LFF was also found in this study. Among the tested fungal pathogens, *C. coccodes* seemed to be most sensitive to the LFF. Six LFF of *Acarospora cervina*, *Cladonia coniocrea*, *Evernia prunastri*, *Lecania hyaline*, *Lecanora Argentata* and *Parmelia*

sulcata showed more than 80% of inhibition in mycelial growth of the pathogen (Table 2).

Unlike the previous results using intact lichen thalli extracts (Gulluce et al., 2006; Halama and Van Halywin, 2004), LFF also exhibited strong antifungal activity against fungal pathogens in this study. Therefore, it will be very interesting to investigate the compounds responsible for 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 with various modes of action. LFF of *Lecania hyalina* and *Lecanora argentata* firstly isolated in this study can be served as novel bioresources to develop new biofungicides alternative to current fungicides to control hot pepper anthracnose pathogenic fungi.

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