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Screening for Antifungal Endophytic Fungi Against Six Plant Pathogenic Fungi

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A total of 187 endophytic fungi were isolated from 11 plant species, which were collected from 11 locations in Korea. Their antifungal activities were screened *in vivo* by antifungal bioassays after they were cultured in potato dextrose broth and rice solid media. Antifungal activity against plant pathogenic fungi such as *Magnaporthe grisea* (rice blast), *Corticium sasaki* (rice sheath blight), *Botrytis cinerea* (tomato gray mold), *Phytophthora infestans* (tomato late blight), *Puccinia recondita* (wheat leaf rust), and *Blumeria graminis* f. sp. *hordei* (barley powdery mildew) was determined *in vivo* by observing the inhibition of plant disease development. Twenty (11.7%) endophytic fungi fermentation broths were able to control, by more than 90%, at least one of the six plant diseases tested. Among 187 liquid broths, the F0010 strain isolated from *Abies holophylla* had the most potent disease control activity; it showed control values of more than 90% against five plant diseases, except for tomato late blight. On the other hand, fourteen (7.5%) solid culture extracts exhibited potent disease control values of more than 90% against one of six plant diseases. The screening results of this study strongly suggested that metabolites of plant endophytic fungi could be good potential sources for screening programs of bioactive natural products.

KEYWORDS: Antifungal metabolite, Endophytic fungi, *In vivo* antifungal bioassays, Plant disease, Plant pathogen

Endophytic fungi form inconspicuous infections in the intercellular spaces, petioles and leaves of healthy plants throughout or nearly throughout their life cycle. Endophytes, in contrast to epiphytes, complete their entire life cycle within their host plant (Strobel and Long, 1997). They are found in almost all kinds of plants, including trees, grass, algae and herbaceous plants. Members of the Ascomycotina, Basidiomycotina and Deuteromycotina, as well as some Oomycetes, have been isolated as endophytes. Endophytic fungi are asymptomatic and may be mutualistic; plants protect and feed endophytes, which produce plant-growth-regulatory, antimicrobial, antiviral or insecticidal substances to enhance the growth and competitiveness of the host in nature (Carroll, 1988). Some endophytic fungi are known as reliable sources of bioactive substances with agricultural and/or pharmaceutical potential, as exemplified by taxol (Stierle *et al.*, 1993; Wang *et al.*, 2000), subglutinol A and B (Lee *et al.*, 1995), and peptide leucinostatin A (Stroble and Hess, 1997). Endophytic fungi are thus expected to be potential sources of new bioactive agents. Few studies have been carried out in this area, though.

Microbial antibiotics have played important roles in the development of new agrochemicals. They have been used either directly or as lead molecules. Microbial metabolites, including blasticidin-S, validamycin and kasugamycin, have been commercialized as fungicides. In addition, two groups of microbial metabolites - strobilurins, which are produced by *Strobilurus tenacellus* (Anke *et al.*, 1977) and *Oudemansiella mucida* (Musilek *et al.*, 1969), and pyrrolnitrins, which are produced by *Pseudomonas pyrrocinia* (Arima *et al.*, 1964) - have been used as lead molecules in the synthesis of fungicides. Fenpiclonil and fludioxonil from pyrrolnitrin, and several fungicides from strobilurins such as azoxystrobin, kresoxim-methyl, metominostrobin, trifloxystrobin, picoxystrobin and pyraclostrobin, have been recently commercialized.

To search for bioactive metabolites from endophytic fungi, we collected various plants in Korea, the majority of which were woody plants, and isolated their endophytic fungi. The current study focused on screening endophytic fungi with antifungal activity against six plant pathogenic fungi.

A broad range of woody plants common to the northeastern part of Korea were collected in 11 locations in Kangwon province, Korea, in August 1999. Barnyard grass (*Echinochloa crus-galli*) and arrow root (*Pueraria thungergiana*) were also collected. The number of samples collected and the fungal isolates are listed in Table 1. Stem and/or twig samples were randomly collected from 3 to 5 healthy mature plants per site at different geographical locations. Individual plants were severed aseptically, put into plastic bags, and kept on ice until further processing.

Fungi were isolated using the surface sterilization method described by Collado *et al.* (1996). The sterilized fragments were plated on a malt extract agar medium [20 g malt extract (Becton and Dickinson Co., Sparks, Maryland, USA), 20 g agar and 1.0 liter distilled water] supplemented with 50 μg/ml chloramphenicol (Sigma Co., St.

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Table 1. Endophytic fungi isolated from various plants in Korea and screening of liquid cultures and solid culture extracts with *in vivo* antifungal activity from endophytic fungi against six plant pathogenic fungi

Source	No. of samples	No. of strains	No. of active strains (%)[a]	
			Liquid cultures	Solid cultures
Abies holophylla	2	9	2 (22)	2 (22)
Acer formosum var. *coreanum*	1	1	0 (0)	0 (0)
Echinochloa crus-galli	3	13	5 (39)	1 (7.7)
Ginkgo biloba	3	22	0 (0)	0 (0)
Larix kaemferi	2	21	2 (9.5)	0 (0)
Lespedeza bicolor	4	22	1 (4.5)	3 (14)
Pinus koraiensis	2	11	1 (9.1)	0 (0)
Pinus densiflora	5	24	5 (21)	1 (4.2)
Pueraria thunbergiana	3	32	4 (13)	2 (6.3)
Quercus acutissima	3	8	0 (0)	0 (0)
Robinia pseudo-acasia	3	24	0 (0)	1 (4.2)
Total	31	187	20 (11)	10 (5.3)

[a]Strains showing a more than 90% *in vivo* antifungal activity against at least one of six plant pathogenic fungi.

Louis, USA), and incubated at 25°C. Thirty-two fragments processed from each plant were placed onto a total of 8 plates (4 fragments per plate). The plates were incubated for a period of up to three weeks. Individual fungal strains were transferred to potato dextrose agar (PDA, Bacto Potato Dextrose Dehydrated; Becton and Dickinson Co.) and further incubated at 25°C for at least two weeks. After checking for purity, each fungal culture was transferred to another agar plate. A total of 187 fungi were isolated from healthy plant stems or twigs.

The strains were cultured in both liquid and solid media for the *in vivo* antifungal bioassay against six plant pathogenic fungi. They were cultured in a potato dextrose broth (PDB; Becton and Dickinson Co.) medium for 14 days at 25°C and 150 rpm. The cultures were centrifuged to remove hyphal mass at 5,000 rpm for 15 min before their antifungal activity was assayed.

As for solid culture, Erlenmeyer flasks (1 liter), each containing 200 g of rice and 120 m*l* of distilled water, were autoclaved twice (with a 24-hr interval) for 30 min each at 121°C. The rice was inoculated with mycelium plugs from a 5-day-old PDA plate of the fungus. The flasks were incubated for 4 weeks at 25°C. The mycelial mass and substrate were mixed with 600 m*l* of methanol and blended thoroughly. After filtration through a Whatman No. 2 filter paper (Whatman International Ltd., Maidstone, England), the filtrate was concentrated to dryness and then dissolved in dimethyl sulphoxide (DMSO) at a rate of 10 g of fresh weight of solid culture per 1 m*l*.

The liquid and solid cultures of the endophytic fungi were tested *in vivo* for antifungal activity against the following diseases, using the methods previously described (Kim *et al.*, 2001): rice blast (*Magnaporthe grisea*); rice sheath blight (*Corticium sasaki*); tomato gray mold (*Botrytis cinerea*); tomato late blight (*Phytophthora infestans*); wheat leaf rust (*Puccinia recondita*) and barley powdery mildew (*Blumeria graminis* f. sp. *hordei*). Rice (*Oryza sativa* cv. Nakdong), tomato (*Lycopersicon esculentum* cv. Seokwang), barley (*Hordeum sativum* cv. Dongbori) and wheat (*Triticum aestivum* cv. Chokwang) plants were grown in vinyl pots (4.5 cm in diameter) in a greenhouse at 25±5°C for 1 to 4 weeks. The potted plant seedlings were sprayed with liquid culture supernatants supplemented with Tween 20 (250 μg/m*l*) and Tween 20 solutions containing a DMSO extract of solid cultures at a final rate of 1%, and were allowed to stand for 24 hrs. The treated plant seedlings were inoculated with spores or mycelial suspensions of 6 plant pathogenic fungi, after which disease severity was assessed 3~7 days after inoculation. The percentage of fungal control was acquired using the following equation:

$$\% \text{ control} = 100[(A - B)/A],$$

in which A = the area of infection (%) on leaves or stems sprayed with Tween 20 solution alone and B = the area of infection (%) on treated leaves or sheaths. Control plants were treated with a Tween 20 solution containing 1% DMSO. Pots were arranged to form a randomized complete block, with two replicates per treatment. The mean value (standard deviation) of the two estimates for each treatment was converted into the percentage of fungal control.

A total of 187 endophytic fungi were isolated from 11 plants in this study (Table 1). Liquid cultures and solid culture extracts of 187 endophytic fungi were tested for *in vivo* antifungal activity against six plant pathogenic fungi. Table 1 exhibits the numbers and percentages of the broths and solid culture extracts that controlled, by more than 90%, at least one of six plant diseases. As for the antifungal activity of liquid cultures, 39%, 22% and 21% of endophytic fungi isolated from *E. crus-galli*, *Abies holophylla* and *Pinus densiflora*, respectively, showed potent antifungal activity against plant pathogenic fungi *in vivo*. None of the endophytic fungi broths isolated from maple (*Acer formosum* var. *coreanum*), ginkgo (*Ginkgo biloba*), oak (*Quercus acutissima*) and acacia (*Robinia pseudo-acasia*) displayed potent *in vivo* antifungal activity. In total, 11% out of 187 isolates exhibited potent disease control activity against at least one of six plant diseases. On the other hand, 5.3% out of 187 solid culture extracts of endophytic fungi showed potent antifungal activity. Twenty-two percent and 14% of the endophytic fungi isolated from *A. holophylla* and bush clover (*Lespedeza bicolor*), respectively, displayed antifungal activity against at least one of 6 plant pathogenic fungi. None of the solid

Table 2. Liquid cultures of endophytic fungi showing potent control activity against six plant diseases[a]

Strain	Source	Control Value (%)[b]					
		RCB[c]	RSB	TGM	TLB	WLR	BPM
F0001	PD[d]	100±0	0±0	21±10	30±14	100±0	8±12
F0010	AH	96±0	90±0	97±1.0	10±14	90±4.7	96±0
F0012	AH	91±2.4	20±0	14±0	30±14	60±9.4	25±12
F0023	PT	93±4.7	63±19	14±0	88±2.8	83±4.7	0±0
F0026	PT	100±0	50±0	35±10	10±10	93±0	0±0
F0047	PD	75±12	0±0	7±10	94±0	20±0	0±0
F0068	PD	16±0	0±0	0±0	90±4.4	0±0	0±0
F0090	PD	90±4.7	50±0	21±10	0±0	73±9.4	0±0
F0091	PD	90±0	10±14	0±0	0±0	20±0	0±0
F0109	PT	90±0	0±0	0±0	12±0	93±93	90±0
F0119	PK	16±0	90±0	7±10	0±0	73±9.4	0±0
F0124	LK	16±0	90±0	0±0	0±0	73±9.4	0±0
F0125	LK	8±12	90±0	14±0	12±0	33±0	0±0
F0135	EC	80±4.7	15±21	14±0	90±0	73±9.4	33±0
F0140	EC	8±12	0±0	7±10	98±0	50±24	0±0
F0141	EC	0±0	0±0	14±20	90±0	3±4.7	0±0
F0142	EC	33±0	0±0	14±0	99±0.9	98±2.4	0±0
F0160	PT	10±0	0±0	0±0	96±0	0±0	0±0
F0168	EC	5±7.1	0±0	14±0	95±1.8	10±14	0±0
F0191	LB	16±0	0±0	71±0	90±0	20±0	0±0

[a]The plant seedlings were incubated with spores or mycelial suspensions of the test organisms 1 day after the culture supernatants of 187 endophytic fungi were sprayed to run off on the leaves.
[b]Each value represents the mean of two replicates±standard deviation.
[c]RCB, rice blast; RSB, rice sheath blight; TGM, tomato gray mold; TLB, tomato late blight; WLR, wheat leaf rust; and BPM, barley powdery mildew.
[d]PD, *Pinus densiflora*; AH, *Abies holophylla*; PT, *Pueraria thunbergiana*; PK, *Pinus koraiensis*; LK, *Larix kaempferi*; EC, *Echinochloa crus-galli*; and LB, *Lespedeza bicolor*.

Table 3. Solid cultures of endophytic fungi showing potent control activity against six plant diseases[a]

Strain	Source	Control Value (%)[b]					
		RCB[c]	RSB	TGM	TLB	WLR	BPM
F0001	PD[d]	0±0	0±0	0±0	63±0	93±0	0±0
F0010	AH	10±0	10±14	0±0	6±8.8	60±9.4	98±0
F0012	AH	0±0	30±0	0±0	0±0	90±4.7	0±0
F0023	PT	5±7.1	0±0	0±0	0±0	90±4.7	0±0
F0111	PT	98±0	0±0	79±5.9	94±0	93±0	90±0
F0168	EC	93±2.4	0±0	63±18	80±±0	20±0	90±0
F0179	RP	90±0	0±0	50±0	0±0	7±0	0±0
F0189	LB	93±2.4	0±0	50±0	75±4.4	80±0	0±0
F0191	LB	38±12	60±14	0±0	0±0	57±4.7	100±0
F0196	LB	0±0	0±0	0±0	0±0	97±0	81±2

The plant seedlings were incubated with spores or mycelial suspensions of the test organisms 1 day after the extracts of rice cultures of 187 endophytic fungi were treated onto the leaves.
[b]Each value represents the mean of two replicates±standard deviation.
[c]RCB, rice blast; RSB, rice sheath blight; TGM, tomato gray mold; TLB, tomato late blight; WLR, wheat leaf rust; and BPM, barley powdery mildew.
[d]PD, *Pinus densiflora*; AH, *Abies holophylla*; PT, *Pueraria thunbergiana*; EC, *Echinochloa crus-galli*; RP, *Robinia pseudo-acasia*; and LB, *Lespedeza bicolor*.

culture extracts of the endophytic fungi from *A. formosum* var. *coreanum*, *G. biloba*, Japanese larch (*Larix kaemferi*), big cone pine (*Pinus koraiensis*) and *Q. acutissima*, however, was active against fungal phytopathogens *in vivo*.

As for the *in vivo* antifungal activity of 187 endophytic fungi broths, culture supernatants of 8, 4, 1, 9, 5, and 2 fungal isolates displayed more than 90% disease control values against rice blast, rice sheath blight, tomato gray mold, tomato late blight, wheat leaf rust and barley powdery mildew, respectively (Table 2). Among 20 active liquid cultures, 15 showed more than 90% disease control values against only one plant disease, and 3 showed the same albeit only against two plant diseases. An F0109 strain from *P. thunbergiana* was highly active against three plant diseases - rice blast, wheat leaf rust and barley powdery mildew - with control values of over 90%. Among 187 liquid broths, the F0010 strain isolated from *A. holophylla* had the most potent disease control activity; it showed control values of more than 90% against five plant diseases, except for tomato late blight. *P. infestans*, which causes tomato late blight, belongs to the Oomycetes, but the other five fungal pathogens are among the so-called "higher fungi", such as Ascomycotina, Deuteromycotina, and Basidiomycotina. Since the evolutionary history of Oomycetes is different from that of higher fungi, they are insensitive to most of the broad-spectrum fungicides currently available. It is therefore likely that the F0010 strain produces broad-spectrum antifungal substances that are active against higher fungi, but not against Oomycetes.

From the results of the screening of solid culture extracts of endophytic fungi, culture extracts of 4, 1, 5, and 4 fungal isolates exhibited potent disease control values of more than 90% against rice blast, tomato late blight, wheat leaf rust and barley powdery mildew, respectively (Table 3). None of the solid culture extracts were highly active against rice sheath blight and tomato gray mold. Among 10 active solid culture extracts, 8 showed disease control values of more than 90% against only one plant disease, and 1 showed the same values but against two plant diseases. An F0111 isolate from *P. thunbergiana* was highly active against four plant diseases, such as rice blast, tomato late blight, wheat leaf rust and barley powdery mildew, with control values of more than 90%. Six endophytic fungi (F0001, F0010, F0012, F0023, F0168 and F0191) produced antifungal substances that are active against fungal pathogens in both liquid and solid media.

Fisher *et al.* (1984) reported that 10 out of 24 isolates of endophytic fungi obtained from 5 species of the Ericaceae grown in a shake culture displayed antimicrobial

activity, and 5 showed both antifungal and antibacterial activities. Pelaez *et al.* (1998) found that there were large differences among isolates from the same species with respect to their ability to produce metabolites with antimicrobial activity, through the screening of 187 endophytic fungi obtained from 9 plant species growing on gypsum and saline soils in central Spain, for the production of antimicrobial activities. In this study, we also found that even if some endophytic fungi looked similar based on colony morphology, they showed varying degrees of *in vivo* antifungal activities against 6 fungal plant pathogens. Recently, Huang *et al.* (2001) reported that 52.3% of endophytic fungi isolated from *Taxus mairei*, *Cephalataxus fortunei* and *Torreya grandis* displayed growth inhibition with respect to at least one pathogenic fungus, such as *Neurospora* sp., *Trichoderma* sp. or *Fusarium* sp. The screening results of previous reports as well as of this study strongly suggest that metabolites of plant endophytic fungi could be good potential sources for screening programs of bioactive natural products. Further study is in progress on the isolation and characterization of antifungal substances produced by the endophytic fungi showing potent disease control activity.

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