J. Microbiol. Biotechnol. (2005), **15**(6), 1402–1407

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

In Vivo Antifungal Effects of *Coptis japonica* Root-Derived Isoquinoline Alkaloids Against Phytopathogenic Fungi

LEE, CHI-HOON, HOI-JOUNG LEE[2], JU-HYUN JEON[1], AND HOI-SEON LEE[1]*

[1]*Faculty of Applied Biotechnology and Research Center for Industrial Development of Biofood Materials, College of Agricultural and Life Science, Chonbuk National University, Chonju 561-756, Korea*
[2]*Division of Life and Genetic Engineering, College of Life and Environmental Sciences, Korea University, Seoul 136-701, Korea*

Received: February 28, 2005
Accepted: March 17, 2005

Abstract The fungicidal activities of *Coptis japonica* (Makino) extracts and their active principles were determined against *Botrytis cineria*, *Erysiphe graminis*, *Phytophthora infestans*, *Puccinia recondita*, *Pyricularia grisea*, and *Rhizoctonia solani* using a whole plant method *in vivo*, and compared with natural fungicides. The responses varied according to the plant pathogen tested. At 2,000 mg/l, the chloroform and butanol fractions obtained from methanolic extracts of *C. japonica* exhibited strong/moderate fungicidal activities against *B. cinerea*, *E. graminis*, *P. recondita*, and *Py. grisea*. Two active constituents from the chloroform fractions and one active constituent from the butanol fractions were characterized as isoquinoline alkaloids, berberine chloride, palmatine iodide, and coptisine chloride, respectively, using spectral analysis. Berberine chloride had an apparent LC_{50} value of approximately 190, 80, and 50 mg/l against *B. cinerea*, *E. graminis*, and *P. recondita*, respectively; coptisine chloride had an LC_{50} value of 210, 20, 180, and 290 mg/l against *B. cinerea*, *E. graminis*, *P. recondita*, and *Py. grisea*, respectively; and palmatine iodide had an LC_{50} value of 160 mg/l against *Py. grisea*. The isoquinoline alkaloids were also found to be more potent than the natural fungicides, curcumin and emodin. Therefore, these compounds isolated from *C. japonica* may be useful leads for the development of new types of natural fungicides for controlling *B. cinerea*, *E. graminis*, *P. recondita*, and *Py. grisea* in crops.

Key words: *Coptis japonica*, berberine chloride, coptisine chloride, palmatine iodide, fungicidal activity

Pre-harvest losses due to fungal diseases in world crop production can amount to 11.8% or even higher in developing countries [1, 4, 14]. Over the past few decades, various attempts to control plant diseases have been made as regards effective eradication or prevention through the development of synthetic fungicides [2]. However, increasing concern over the environmental effects of the currently used synthetic fungicides has highlighted the need for the development of alternative types of selective control or methods of crop protection with or without a reduced use of conventional fungicides [4, 10]. Therefore, research on natural plant products for agriculture has recently intensified as it becomes evident that plant-derived fungicides have enormous potential to influence modern agrochemical research [4]. Although it is difficult to define the ecological significance of most synthetic fungicides, there is good reason to suppose that a secondary plant metabolism has evolved to protect against attack from microbial pathogens [4].

Plant extracts or phytochemicals provide an attractive alternative to currently used synthetic fungicides as regards controlling phytopathogenic fungi, since they constitute a rich source of bioactive chemicals [14, 15], are often active against a limited number of specific target species, are biodegradable into nontoxic products, and are potentially suitable for use in integrated management programs, allowing for the development of new classes of possibly safer disease control agents. Thus, recent efforts have focused on secondary metabolites as potentially useful products for commercial fungicides or lead compounds [3, 7, 16]. The present authors already reported and confirmed that among 53 oriental medicinal plant extracts tested, the extract of *Coptis japonica* roots exhibits potent fungicidal activities against *Botrytis cinerea*, *Botryospaeria dothidea*, *Collectotrichum dematium*, *Erysiphe graminis*, *Fusarium oxysporum*, *Phytophthora capsici*, and *Pyricularia grisea* [16]. Yet *C. japonica* is not only an important source of natural fungicides, but is also known for its

*Corresponding author
Phone: 82-63-270-2544; Fax: 82-63-270-2550;
E-mail: hoiseon@chonbuk.ac.kr

medicinal properties, including antibacterial, antitumor, and antiinflammatory effects [9–13]. However, in spite of its excellent biological activities, *C. japonica* has received little attention in terms of fungal management or damage [9–13, 16]. Accordingly, this study assessed the fungicidal activities of *C. japonica* root-derived isoquinoline alkaloids and the natural fungicides curcumin and emodin against six phytopathogenic fungi [7, 9].

Chemicals, Plant Preperation, and Phytopathogenic Fungi

The curcumin and emodin were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade. The *Coptis japonica* roots (4.0 kg) purchased as a commercially available product were dried in an oven at 45°C for 2 days, finely powdered, extracted twice with methanol (20 l) at room temperature, and filtered (Toyo filter paper No. 2). The filtrate was concentrated at 40°C to yield 740 g of extract (18.5%). The extract was then sequentially partitioned into hexane (54.2 g), chloroform (40.7 g), ethyl acetate (13.5 g), butanol (134.1 g), and water-soluble (499.5 g) portions for the subsequent bioassay with phytopathogenic fungi. The organic solvent portions were concentrated to dryness using a rotary evaporator at 40°C, while the water portion was freeze-dried.

The six plant diseases evaluated in this study were rice blast, rice sheath blight, cucumber gray mold, tomato late blight, wheat leaf rust, and barley powdery mildew caused by *Pyricularia grisea*, *Rhizoctonia solani*, *Botrytis cinerea*, *Phytophthora infestans*, *Puccinia recondite*, and *Erysiphe graminis*, respectively. Except for *P. recondita* and *E. graminis*, the others were routinely maintained on potato dextrose agar (PDA) slants and V-8 agar slants, and kept for stock at 4°C.

Isolation and Identification

The chloroform fraction (14 g) was chromatographed on a silica gel column (70–230 mesh, 600 g, 5.5 i.d.×70 cm Merck, Darmstadt, Germany), and successively eluted with a stepwise gradient of chloroform/methanol (0, 5, 10, 20, 30, 50, and 100%). The active fraction (4.1 g) was chromatographed on a silica gel column and eluted with chloroform/methanol (10:1). The column fractions were then analyzed by thin layer chromatography (TLC) (chloroform/methanol, 10:1), and fractions with similar TLC patterns combined. For further separation of the biologically active substances, preparative high-performance liquid chromatography (HPLC, Delta Prep 4000, Waters, Milford, MA, U.S.A.) was used. The column was a Bondapak C18 (2.9 i.d.×300 mm, Waters) using methanol/water (6:4) at a flow rate of 7 ml/min and detection at 254 nm. The chemical substances in the active peak (58 mg) among three peaks showed two main yellow spots on TLC, which were then developed by benzene/ethyl acetate/*n*-propanol/methanol/ethylamine (8:5:2:1:1). The two spots became an orange-red color when reacted with the Dragendorff reagent, suggesting that the chemical substances were alkaloids. The active fraction was then chromatographed on a silica gel column using the previous solvent system to give fractions 1 and 2, and the active compounds isolated according to the method of Lee [12]. Fractions 1 (108 mg) and 2 (136 mg) were dissolved in water, and precipitates obtained from the aqueous solution by adding 1 N HCl to pH 5.0 and a 7.5% HI solution to pH 4.0, respectively. The precipitates were collected by filtration, washed with water, and dried under reduced pressure over P_2O_5. The yield from fractions 1 and 2 was 75.8 mg (Compound I) and 91.3 mg (Compound II), respectively. The Rf values for compounds I and II were 0.67 and 0.43 in benzene/ethyl acetate/*n*-propanol/methanol/ethylamine (8:5:2:1:1), respectively.

The butanol fraction (14 g) was chromatographed on a silica gel column and eluted with benzene/ethyl acetate/*n*-propanol/methanol/ethylamine (8:5:2:1:1). The active fraction (2.6 g) was chromatographed on a silica gel column and eluted with butanol/acetone/water (4:1:1). The column fractions were analyzed by TLC (butanol/acetone/water, 4:1:1), and fractions with similar TLC patterns combined. The chemical substances in the active fraction among three fractions exhibited a main yellow spot on the TLC, which was then developed by benzene/ethyl acetate/*n*-propanol/methanol/ethylamine (8:5:2:1:1). For further separation of the biologically active substance, preparative HPLC was used, as mentioned above. Finally, the active principle (111 mg, Compound III) was isolated, and the R_f value for the isolate was 0.98 in the previous solvent system.

The structural determination of the active isolates was made using a spectral analysis. The ^{1}H- and ^{13}C-NMR spectra were recorded using a Bruker AM-500 spectrometer, the UV spectra using a Waters 490 spectrometer, the IR spectra using a Biorad FT-80 spectrophotometer, and the mass spectra using a JEOL JMS-DX 30 spectrometer.

Bioassay and Statistical Analysis

The fungicidal activities of the test samples were determined using a whole plant method in a greenhouse, as previously described [9, 10]. The initial concentration of the test solution was 2,000 mg/l, at which over 60% of the compounds exhibited fungicidal activity against the 6 test fungi; further tests were employed with a dilution sequence of 1,000, 500, 250, 125, and 62.5 mg/l.

To prepare test solutions at a concentration of 2,000 mg/l, 100 mg of each test sample was dissolved in 0.5 ml of dimethyl sulfoxide (DMSO), followed by dilution with 49.5 ml of water containing Tween 20 (250 μg/ml).

Each test sample solution (50 ml) was sprayed onto two pots on a turntable at the same time. The treated plants were then kept in a greenhouse for 1 day, before being inoculated by each pathogen. The controls were just sprayed with the Tween 20 solution. All tests were replicated three times.

For the test with rice blast (RCB) caused by *Py. grisea*, rice plants in the 2nd leaf stage (three plants/pot) were sprayed with each test solution. The treated plants were then inoculated with a suspension of conidia in distilled water (1×10^6 spores/ml) and kept in a chamber (25°C) for 24 h under 100% relative humidity (RH). Thereafter, the treated and control plants were placed in a low lighted chamber (26±2°C and 85% RH) for 5 days, and rated for the disease severity. For the rice sheath blight (RSB) caused by *R. solani*, each test solution was sprayed onto rice plants in the 3rd leaf stage (three plants/pot). The plants were then inoculated by injecting the inoculum at the base of the rice plants. The inoculum of *R. solani* was made by inoculating mycelial plugs in a wheat bran medium at 25°C for 7 days, then macerating at a ratio of 500 g of medium-incubated *R. solani* per 1 l of distilled water into the mixer. Thereafter, the treated and control plants were placed in a lighted chamber (28°C) for 5 days. For the cucumber gray mold (CGM) caused by *B. cinerea*, cucumber plants in the 1st leaf stage (one plant/pot) were sprayed with each test solution. The plants were then inoculated with conidia (1×10^6 spores/ml) of *B. cinerea* incubated on a PDA medium at 20°C for 15 days using a leaf spray and placed in a chamber (20°C) for 4–5 days. For the tomato late blight (TLB) caused by *P. infestans*, each test solution was sprayed onto tomato plants in the 2nd leaf stage (two plants/pot). The plants were then inoculated with a suspension of 1×10^5 zoosporangia/ml made from a 14-day culture of a V-8 juice agar medium at 20°C, placed in a chamber (18°C) for 4 days, and rated for the disease severity. For the wheat leaf rust (WLR) caused by *P. recondita*, wheat plants in the 1st leaf stage (four plants/pot) were sprayed with each test solution. The plants were then sprayed with a suspension (60 mg/100 ml of 250 ppm Tween 20) of uredospores collected from the 2nd leaf stage of wheat, and placed in a moist chamber. One day after inoculation, the plants were moved to a growth chamber (20°C and 70% RH). The fungicidal activities of the test samples were evaluated 10 days after inoculation (DAI). For the barley powdery mildew (BPM) caused by *E. graminis*, barley plants with a fully expended first leaf (four plants/pot: f 7.5 cm) were sprayed with a suspension of the test material. The treated plants were then dusted with *E. graminis* conidia formed on the leaves of barley based on a ratio of 8 test pots/one control pot.

The control effect of the test samples on each disease was evaluated using a control value (CV) calculated by the formula CV (%)=[(A−B)/A]×100, where A and B represent the disease area on the untreated and treated plants, respectively. The LC_{50} values were calculated using a probit analysis, whereas an analysis of variance was performed with the PROC GLM procedure (SAS institute, Cary, NC, U.S.A.). If $P>F$ was less than 0.01, the means were separated using the least significant difference (LSD) pest at the level $P=0.05$.

Table 1. Antifungal activities of solvent fractions of *C. japonica* extracts against phytopathogenic fungi[a].

Material	Conc. (mg/l)	Control values (%)[b]					
		RCB	RSB	CGM	TLB	WLR	BPM
Methanol extract	2,000	92	0	70	0	100	100
	1,000	70	0	45	0	82	78
Hexane fraction	2,000	0	0	0	0	0	0
	1,000	0	0	0	0	0	0
Chloroform fraction	2,000	100	0	74	0	100	85
	1,000	78	0	48	0	76	69
Ethyl acetate fraction	2,000	0	0	0	0	0	0
	1,000	0	0	0	0	0	0
Butanol fraction	2,000	50	0	68	0	80	100
	1,000	24	0	39	0	62	86
Water fraction	2,000	0	0	0	0	0	67
	1,000	0	0	0	0	0	41
LSD (0.05)[c]		9.9	-	7.3	-	9.3	9.5

[a]RCB, rice blast caused by *Pyricularia grisea* in rice plants; RSB, rice sheath blight caused by *Rhizoctonia solani* in rice plants; CGM, cucumber gray mold caused by *Botrytis cinerea* in cucumber plants; TLB, tomato late blight caused by *Phytophthora infestans* in tomato plants; WLR, wheat leaf rust caused by *Puccinia recondita* in wheat; BPM, barley powdery mildew caused by *Erysiphe graminis* in barley.
[b]Control value (%)=100×{disease severity in untreated plants−disease severity in treated plants}÷disease severity in untreated plants.
[c]LSD, least significant difference.

Table 2. Antifungal activities of components isolated from *C. japonica* and natural fungicides against phytopathogenic fungi[a].

Material	Conc. (mg/l)	control values (%)[b]					
		RCB	RSB	CGM	TLB	WLR	BPM
Berberine chloride	500	0	0	76	0	100	100
	250	0	0	65	0	100	83
	125	0	0	30	0	80	68
	62.5	0	0	0	0	54	45
	LC_{50} (mg/l)	-	-	190	-	50	80
Coptisine chloride	500	57	0	62	0	78	100
	250	40	0	48	0	66	100
	125	18	0	28	0	33	90
	62.5	0	0	0	0	10	75
	LC_{50} (mg/l)	290	-	210	-	180	20
Palmatine iodide	500	100	0	0	0	0	0
	250	72	0	0	0	0	0
	125	45	0	0	0	0	0
	62.5	25	0	0	0	0	0
	LC_{50} (mg/l)	160	-	-	-	-	-
Dichlofluanid[c]	50	0	0	91	0	0	0
Curcumin	LC_{50} (mg/l)	-	260	540	190	90	-
Emodin	LC_{50} (mg/l)	-	100	160	380	-	50
LSD (0.05)[d]		9.1	-	6.8	-	8.9	9.5

[a]RCB, rice blast caused by *Pyricularia grisea* in rice plants; RSB, rice sheath blight caused by *Rhizoctonia solani* in rice plants; CGM, cucumber gray mold caused by *Botrytis cinerea* in cucumber plants; TLB, tomato late blight caused by *Phytophthora infestans* in tomato plants; WLR, wheat leaf rust caused by *Puccinia recondita* in wheat; BPM, barley powdery mildew caused by *Erysiphe graminis* in barley.
[b]Control value (%)=100×{disease severity in untreated plants−disease severity in treated plants}÷disease severity in untreated plants.
[c]Commercial name.
[d]LSD, least significant difference.

During the initial experiments, the methanolic extract of the *Coptis* roots exhibited significant fungicidal activities (>90% control values) against *E. graminis*, *P. recondita*, and *Py. grisea* and moderate fungicidal activities (>70% control values) against *B. cinerea* at a concentration of 2,000 mg/l. However, this methanolic fraction showed no fungicidal activity against *R. solani* and *P. infestans*. Further solvent fractionation of the methanolic extract of the *Coptis* roots revealed strong fungicidal activities by the resulting chloroform fraction against *B. cinerea*, *E. graminis*, *P. recondita*, and *Py. grisea* with 74, 85, 100, and 100% control values, respectively, and by the butanol fraction against *B. cinerea*, *E. graminis*, *P. recondita*, and *Py. grisea* with 68, 100, 80, and 50% control values, respectively, at a concentration of 2,000 mg/l (Table 1). However, minimal fungicidal activity was exhibited by the ethyl acetate and water fractions.

Because of the strong activity of the chloroform and butanol fractions, purification of the biologically active compounds from these fractions was achieved using silica gel column chromatography and HPLC, and then the isolates were bioassayed. Two and one active principles were isolated from the chloroform and butanol fractions, respectively, and identified as isoquinoline alkaloids based on the color reaction with the Dragendorff reagent. All three active isolates exhibited potent fungicidal activities against *B. cinerea*, *E. graminis*, *P. recondita*, and *Py. grisea* (Table 2). The structural determination of the isolates was obtained using spectroscopic methods including MS and NMR and by direct comparison with authentic reference compounds, resulting in the identification of the isoquinoline alkaloids berberine chloride, coptisine chloride, and palmatine iodide. The ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra for the berberine chloride and palmatine iodide isolated from the chloroform fraction, and coptisine chloride isolated from the butanol fraction, were found to be the same as those for the berberine chloride, palmatine iodide, and coptisine chloride isolated from *C. japonica* roots [5].

The fungicidal activities of berberine chloride, palmatine iodide, and coptisine chloride against the six phytopathogenic fungi when treated at various concentrations were determined *in vivo*. Berberine chloride exhibited strong/moderate fungicidal activities against *E. graminis* and *P.*

recondita and moderate/weak fungicidal activities against *B. cinerea* at 500, 250, 125, and 62.5 mg/l, yet no activity against the rice blast (RCB) caused by *Py. grisea*, rice sheath blight caused by *R. solani*, and tomato late blight caused by *P. infestans*. Meanwhile, coptisine chloride exhibited strong/moderate fungicidal activities against *E. graminis* at 500, 250, 125, and 62.5 mg/l and moderate fungicidal activities against *B. cinerea*, *P. recondita*, and *Py. grisea* at 500 and 250 mg/l, yet no activity against the tomato late blight caused by *P. infestans* and rice sheath blight caused by *R. solani*. Finally, palmatine iodide exhibited strong/moderate fungicidal activity against *Py. grisea* at 500, 250, and 125 mg/l. Berberine chloride had an apparent LC_{50} value of approximately 190, 80, and 50 mg/l against *B. cinerea*, *E. graminis*, and *P. recondita*, respectively; coptisine chloride had an LC_{50} value of 210, 20, 180, and 290 mg/l against *B. cinerea*, *E. graminis*, *P. recondita*, and *Py. grisea*, respectively; and palmatine iodide had an LC_{50} value of is 160 mg/l against *Py. Grisea* (Table 2). As such, this is the first report on the fungicidal function of these components isolated from *C. japonica* roots against *B. cineria*, *E. graminis*, *P. infestans*, *P. recondita*, *Py. grisea*, and *R. solani*. In the present study, the fungicidal activity of the *Coptis* materials including isoquinoline alkaloids was much more pronounced in *B. cinerea*, *E. graminis*, *P. recondita*, and *Py. grisea*, than in *R. solani* and *P. infestans*. It has already been reported that *Coptis* root-derived isoquinoline alkaloids have antibacterial, antidiabetic, antitumor, antidiarrhoea, and antiinflammatory effects [5, 9–13], and among the constituents of *C. japonica*, isoquinoline alkaloids constitute a major group of secondary metabolites [5, 10, 11]. *C. japonica* root-derived materials have also been reported to exhibit herbicidal activities against *Agrostis palustris* and *Lemna minor* [6], whereas isoquinoline alkaloids (berberine chloride, palmatine iodide, and coptisine chloride) have an inhibitory effect on *Bifidobacterium longum*, *B. bifidum*, *Clostridium perfringens*, and *C. paraputrificum*, yet a minimal or no effect on *B. adolescentis*, *Lactobacillus acidophilus*, *L. casei*, and *Escherichia coli* [5].

Because of the fungicidal activities of berberine chloride, palmatine iodide, and coptisine chloride against the six phytopathogenic fungi, the compounds were compared with the natural fungicides curcumin and emodin (Table 2). Curcumin exhibited strong fungicidal activities against *B. cineria*, *P. infestans*, *P. recondita*, and *R. solani*, yet did not inhibit the growth of *E. graminis* and *Py. grisea*. In addition, curcumin had an apparent LC_{50} value of 540, 190, 90, and 260 mg/l against *B. cineria*, *P. infestans*, *P. recondita*, and *R. solani*, respectively. Meanwhile, emodin revealed strong fungicidal activities against *B. cineria*, *E. graminis*, *P. infestans*, and *R. solani*, yet did not inhibit the growth of *P. recondita* and *Py. grisea*. Emodin had an apparent LC_{50} value of 160, 50, 380, and 100 mg/l against *B. cineria*, *E. graminis*, *P. infestans*, and *R. solani*, respectively. Therefore, although the fungicidal activities of berberine chloride, coptisine chloride, and palmatine iodide against *B. cineria* were less than that of synthetic fungicides such as dichlofluanid, the isoquinoline alkaloids were still comparable with natural fungicides, such as curcumin and emodin. Accordingly, these compounds may be useful leads for developing new types of natural fungicides for controlling *B. cinerea*, *E. graminis*, *P. recondita*, and *Py. grisea* on crops.

In conclusion, the present results indicated that *Coptis japonica* root-derived isoquinoline alkaloids exhibit fungicidal effects *in vivo* against *B. cinerea*, *E. graminis*, *P. recondita*, and *Py. Grisea*, making them useful leads in the development of safer disease control agents.

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