Effect of Oriental Medicinal Plant Extracts on Spore Germination of *Alternaria brassicicola* and Nature of Inhibitory Substances from Speedweed

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ABSTRACT

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Among 65 species of oriental medicinal plants tested, 39 contained substances inhibitory to conidial germination of *Alternaria brassicicola*, with the most inhibitory extract from speedweed (*Polygonum perfoliatum*). The inhibitory substances in dried speedweed were insoluble in water. However, the inhibitors were readily extracted with ethanol or methanol, but not with acetone, ether, or chloroform. The ethanol extract was very effective in controlling black leaf spot of spoon cabbage, *Brassica campestris* subsp. *chinensis*, caused by *A. brassicicola*. The inhibitory effect of the extract was not affected by treatment with anion exchange resins, but was partially reduced by cation exchange resins, indicating the presence of two inhibitory substances in the extract, one with a positive charge and the other with no charge on its molecule. The inhibitory substances in the extract were dialyzable with molecular weight minimums of 10,000 or 1,000, but not 500 or 100, suggesting that both inhibitors have molecular weights between 500 and 1,000.

Additional keyword: iprodione

Leaf spots of crucifers caused by *Alternaria brassicicola* (Schwein) Wiltshire, *A. brassicae* (Berk.) Sacc., and *A. raphani* (J.W. Groves & Skolko) are worldwide in distribution (5). These pathogens have caused considerable losses in cruciferous oil production and vegetable crops yield in different parts of the world (10,16,18). In Taiwan, *A. brassicicola*, the main causal organism of leaf spot, is widespread on the island, occurring all year round, and causes reduction in quality and yield of cruciferous vegetables.

Control of foliar plant diseases relies heavily upon the use of chemical fungicides (1,8), but high concern for adverse effects of pesticides on human health and the environment requires development of alternative methods for disease control. Extracts from a number of plant species are known to contain substances inhibitory to fungi (6,19), and many essential oils

show antifungal activity against a wide range of fungi (7,20). The extracts from *Reynoutria sachalinensis* (F. Schmidt) Nakai (17) and sunflower oil (15) also have been developed for use by growers for control of powdery mildews.

A project was initiated to test the possibility of using extracts of edible plants for control of plant pathogens. Because some Chinese herbs have been used in treating infection by microorganisms (2,9), they were tested for ability to control powdery mildews. Results showed that more than 21% of dried Chinese herbs purchased from a Chinese herb store contained substances in their aqueous extracts inhibitory to spore germination of *Oidium murrayae* Hosag., U. Braun & Rabindram and that extracts from Chinese rhubarb (*Rheum officinale* Baill.) and Japanese knotweed (*Polygonum cuspidatum* Sieb & Zucc.) were very effective in controlling powdery mildews on cucumber, pumpkin, and eggplant (3). A large number of oriental medicinal plants has been collected and planted in recent years in a special nursery at Taitung District Agricultural Improvement Station, Taitung, Taiwan. Extracts were prepared from these plants and tested for inhibition of conidial germination of *A. brassicicola*. The most inhibitory extract from this bioassay was tested for control of black leaf spot and partially characterized.

MATERIALS AND METHODS

Source of spores. *A. brassicicola* was isolated from *Brassica oleracea* L. and identified based on its distinct morphological characteristics of conidia (4). For preparation of conidial suspension, the fungus was grown on 10% V8 agar (10% V8 juice, 0.02% CaCO₃, and 2% agar) at 24°C in a 12-h light-and-dark cycle, with cool white fluorescent irradiation. A conidial suspension was prepared by transferring conidial mass from a 2-week-old culture with a transfer loop into 9 ml of sterile distilled water, and the concentration of the suspension was adjusted to approximately 10⁵ spores/ml by a Pipetman microliter pipet (West Coast Scientific, Oakland, CA; 12).

Preparation of plant extracts. Medicinal plants used in this study were collected from the medicinal plant nursery of Taitung District Agricultural Improvement Station, Taitung, Taiwan. The aboveground parts, including stems, leaves, flowers, and fruit, if they were present at the time of collection, were washed with tap water, air dried, and cut into pieces approximately 5 cm in length. Extracts were obtained by grinding and pressing these pieces of plant tissues with a juice extractor (Paiyun Co., Taipei, Taiwan) and centrifuged at 6,000 rpm for 15 min, twice. Supernatants were sterilized by filtration through a 0.22-μm Millipore filter (Millipore Co., Billerica, MA).

Effects of extracts on spore germination. To test plant extracts for inhibition of spore germination, 10 μl of spore suspension of *A. brassicicola* was mixed with 10 μl of extract in a cavity of a sterile eight-cavity slide. Slides with spores were kept moist by placing them on L-shaped glass rods on moistened paper towels in large petri dishes (150 by 20 mm) sealed with Parafilm. Germination was recorded after incubation at 24°C for 8 h, and 100 spores were counted in each of four replicates. All the experiments were repeated twice. Data presented were means from one of the experiments, with both experiments showing similar results.

Extraction with different solvents and effect on spore germination. Speedweed (*P. perfoliatum* L.) plants were collected at the campus of National Pingtung University of Science and Technology, washed with tap water, dried in an oven at 50°C for

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24 h, and cut into pieces about 1 to 3 cm in length. Extraction was carried out by shaking 10 g of tissue with either 100 ml of water, 50% ethanol, 95% ethanol, methanol, acetone, ether, or chloroform in a 250-ml flask on a shaker at 150 strokes/min for 24 h. After filtration, the extract was adjusted to 100 ml with the same solvent. The extract (5 ml) was evaporated to dryness in the fume hood, and the residue was redissolved in 5 ml of 50% ethanol. The solution was diluted 100-, 250-, 500-, and 1,000-fold with sterile distilled water before being used for germination tests. Ethanol similarly diluted with distilled water was used as the control. The experiments were performed twice.

Control of black leaf spot with speedweed extract. Seeds of spoon cabbage (*B. chinensis* L. cv. Ching-green) were purchased from Known-You Seed Co., Kaohsiung, Taiwan and grown in 250-ml pots containing a mixture of nine parts peat moss and one part vermiculite. Three-week-old plants, each with four to five fully expanded leaves, were sprayed to runoff with 50% ethanol extract of speedweed at 500- and 1,000-fold dilution. For comparison, plants were similarly sprayed with iprodione (50%, WP), a fungicide inhibitory to *A. brassicicola* (10) at a 2,000-fold dilution recommended by the manufacturer (Sinon Co., Taichung, Taiwan). Control plants were sprayed with 50% ethanol diluted 500-fold. After being air dried on the bench, each leaf was inoculated with five 1- μ l drops of spore sus-

pension of *A. brassicicola* along the edge of leaf, and a 15- μ l drop of 1% water agar at 60 to 70°C was added to each inoculum drop (21). Inoculated plants were placed in moist chambers and kept in the laboratory at 24°C. The number of lesions that developed at the inoculated sites was recorded 3 days after inoculation. Three leaves per plant were inoculated and three plants were used for each treatment. The experiment was repeated once.

Characterization of inhibitors in speedweed extract and effect on spore germination. To study the effects of different treatments on the inhibitory property of speedweed extract, 10 g each of Dowex anion exchange resins (Dow Chemical Co., Midland, MI), Lowatit cation exchange resins (Bayer Corporation, Pittsburgh), or activated charcoal (Taipei Chemical Industry Co., Hsinchu, Taiwan) were washed with 100 ml of distilled water three times by shaking for a 6-h period to remove possible inhibitory substances (13). Then, 50 ml of 50% ethanol extract of speedweed at 250-fold dilution was shaken with either 5 g of each resin or 5 g of activated charcoal in a 250-ml flask at 150 strokes/min for 24 h and filtered through a Whatman no. 1 filter paper. The treated extracts were diluted 750-, 1,000-, and 1,250-fold with sterile distilled water before being used for germination tests.

To estimate the molecular weight of the inhibitory substances, 5 ml of 50% ethanol extract diluted 200-fold was placed in a molecular porous membrane tubing with

molecular weight minimum of 100, 500, 1,000 or 10,000, and dialyzed at 24°C against 1,500 ml of distilled water changed three times over a 24-h period. The treated extracts were diluted 750-, 1,000-, and 1,250-fold with sterile distilled water before being used for germination tests.

Four replicates were used for each treatment and all the experiments were repeated twice with similar results. Data presented were means from one of the experiments.

RESULTS

Effect of extracts obtained from medicinal plants on spore germination. Among the 65 extracts from macerated medicinal plants, 24 contained substances capable of complete inhibition of conidial germination of *A. brassicicola* (Table 1). Nine extracts reduced the germination rate to 3 to 38%, and six extracts by 60 to 69%. Twenty-six extracts were slightly to non-inhibitory to *A. brassicicola*, with 72 to 94% germination. When the 24 inhibitory extracts were tested at 50-fold dilution, 6 showed complete inhibition of conidial germination of *A. brassicicola*, whereas 18 other extracts were slightly to non-inhibitory to *A. brassicicola*, with 74 to 94% germination (Table 2). These six inhibitory extracts then were tested at 100-, 500-, 1,000-, and 2,000-fold dilutions. Speedweed extract was the most inhibitory to *A. brassicicola*, completely inhibiting germination at 2,000-fold dilution (Table 3), and was selected for further studies. Extracts of Japanese knotweed, jimsonweed, and autumn maple were inhibitory to *A. brassicicola* at 100-fold dilution but not at 500-fold dilution, while those of Chinese knotweed and Taiwanese euphorbia were not inhibitory at 100-fold dilution.

Extraction of the inhibitory substances from speedweed. Of the solvents tested, ethanol at 50% was the most effective in extracting the substances inhibitory

Table 1. Effect of extracts of medicinal plants on germination of conidia of *Alternaria brassicicola*^y

Plant no.	Scientific name	Common name	Germination (%) ^z
1	<i>Allium fistulosum</i>	Green onion	0
2	<i>Allium sativum</i>	Garlic	0
3	<i>Allium tuberosum</i>	Chinese chives	0
4	<i>Aster altaicus</i>	...	0
5	<i>Averrhoa bilimbi</i>	Cucumber tree	0
6	<i>Belamcanda chinensis</i>	Leopard lily	0
7	<i>Bidens pilosa</i>	Hairy beggarticks	0
8	<i>Bischofia javanica</i>	Autumn maple	0
9	<i>Cedrela sinensis</i>	Chinese cedar	0
10	<i>Chamaesyce hirta</i>	Asthma plant	0
11	<i>Datura stramonium</i>	Jimsonweed	0
12	<i>Derris trifoliata</i>	Asi-asiman	0
13	<i>Euphorbia formosana</i>	Taiwanese euphorbia	0
14	<i>Euphorbia tirucalli</i>	Pencil tree	0
15	<i>Fructus ligustri</i>	Glossy privet fruit	0
16	<i>Garcinia spicata</i>	Common garcinia	0
17	<i>Lygodium japonicum</i>	Climbing fern	16
18	<i>Oxalis corniculata</i>	Yellow wood sorrel	0
19	<i>Phyllanthus urinaria</i>	Shatterstone	0
20	<i>Plumeria rubra</i>	Plumeria	0
21	<i>Pogonatherum crinitum</i>	German grass	3
22	<i>Pogostemon cablin</i>	Patchouli	0
23	<i>Polygonum chinense</i>	Chinese knotweed	0
24	<i>Polygonum cuspidatum</i>	Japanese knotweed	0
25	<i>Polygonum perfoliatum</i>	Speedweed	0
26	<i>Rhinacanthus nasutus</i>	...	0
27	<i>Talinum triangulare</i>	Mikado pheasant	18
28	Water (control)	...	95

^y Spore suspension mixed with the same amount of extract was incubated at 24°C for 8 h.

^z Four replicates were used for each plant species and 100 spores were counted for each replicate. Percentage of germination was based on the average of two tests.

Table 2. Effect of extracts of 24 species of medicinal plants from the first selection on germination of conidia of *Alternaria brassicicola* at 50-fold dilution

Source of extract ^z	Germination (%)
Speedweed	0
Autumn maple	0
Chinese knotweed	0
Japanese knotweed	0
Jimsonweed	0
Taiwanese euphorbia	0
Water (control)	94

^z Species with extract slightly or non-inhibitory to *A. brassicicola*: *Rhinacanthus nasutus*, patchouli, common garcinia, yellow wood sorrel, garlic, hairy beggarticks, cucumber tree, Chinese chives, glossy privet fruit, green onion, asi-asiman, plumeria, asthma plant, pencil tree, leopard lily, *Aster altaicus*, Chinese cedar, and shatterstone.

to spore germination of *A. brassicicola* from dried speedweed tissue, followed by methanol and 95% ethanol (Table 4). Water, acetone, ether, and chloroform were ineffective in extracting the inhibitory substances with the methods used. Subsequently, inhibitory substances obtained by extracting dried speedweed tissue with 50% ethanol were used for disease control and inhibitor characterization.

Disease control with speedweed extract. Ethanol extract of speedweed was very effective in controlling black leaf spot of spoon cabbage caused by *A. brassicicola*, reducing the spot incidence from 100% on the control to 13% on plants sprayed with extract at 500-fold dilution (Table 5). Even at 1,000-fold dilution, the extract was able to reduce the leaf spore development to 33%. The fungicide iprodione reduced the spot incidence to 0%.

Nature of inhibitory substances. At 2,500-fold dilution, the speedweed extract completely inhibited the conidial germination of *A. brassicicola*. The extract was no longer inhibitory to spore germination after treatment with activated charcoal (Table 6). After treatment with cation exchange resins, the extract became non-inhibitory at 2,500-fold dilution, but was still strongly inhibitory at the 2,000-fold dilution with only 15% germination of treated conidia. Repeated treatment with fresh cation exchange resins did not further reduce the inhibitory effect (*data not shown*). Treatment with anion exchange resins did not affect the inhibitory effect of the extract.

The inhibitory substances in the speedweed extract did not diffuse through the dialysis membrane with molecular weight minimum of 100 or 500 (Table 7). However, the extract in the dialysis tubing with molecular weight minimum of 1,000 or 10,000 was no longer inhibitory to spore germination of *A. brassicicola* following dialysis treatment.

Table 3. Effect of extract dilutions of six medicinal plants on germination of *Alternaria brassicicola* conidia

Source of extract ^z	Germination (%) at <i>n</i> -fold dilution ^y		
	100	500	1,000
Speedweed	0	0	0
Japanese knotweed	0	72	93
Jimsonweed	0	71	92
Autumn maple	0	93	93
Chinese knotweed	95	94	94
Taiwanese euphorbia	96	97	91
Water (control)	94	92	94

^y All plants listed are those that effectively inhibited conidial germination at 50-fold dilution. Data are mean of four replicates from one of the experiments with similar results.

^z Spore suspension mixed with the same amount of extract was incubated at 24°C for 8 h.

DISCUSSION

Of the 65 species of oriental medicinal plants tested in this study, 60% contained substances inhibitory to spore germination of *A. brassicicola* and nearly 40% were strongly inhibitory. Although, following dilution, most of the extracts from macerated plants were only moderately or slightly inhibitory, the amount of the fungitoxic substances extracted may be substantially increased when different extraction methods are used (14) or if only

certain parts of plants are used for extraction. The extract from speedweed was found to be highly effective in controlling black leaf spot of spoon cabbage caused by *A. brassicicola*. These results suggest that oriental medicinal plants are a good material source for obtaining desirable substances for controlling leaf spots of crucifers and possibly other plant diseases as well.

Dried whole plants of speedweed have been shown to have anticancerous properties (9,11). Our results show that speed-

Table 4. Effectiveness of solvents to extract inhibitory substances from speedweed to prevent conidial germination of *Alternaria brassicicola*^z

Solvent	Germination (%) at <i>n</i> -fold dilution ^z			
	200	500	1,000	2,000
Water	89	95	96	95
Ethanol (50%)	0	0	0	0
Ethanol (95%)	0	0	87	95
Methanol	0	0	0	97
Acetone	74	96	96	95
Ether	98	95	96	96
Chloroform	97	97	97	97
Control (50% ethanol)	99	nt	nt	nt

^y Leaves, stems, flowers, and fruit of the speedweed plant were used to obtain the extract.

^z Spore suspension mixed with the same amount of extract was incubated at 24°C for 8 h. Data are mean of four replicates; nt = not tested.

Table 5. Comparison of speedweed extracts with iprodione for control of black leaf spot of spoon cabbage caused by *Alternaria brassicicola*

Treatment	Spot incidence (%) ^z
Speedweed extract (500-fold dilution)	13 B
Speedweed extract (1,000-fold dilution)	33 C
Iprodione	0 A
Control (50% ethanol at 500-fold dilution)	100 D

^z Each of the three leaves on each plant was inoculated at five locations, and three plants were used for each treatment. Spot development was recorded 3 days after inoculation at 24°C with 12 h of light. Values followed by the same letter are not significantly different by Tukey's significant difference test ($\alpha = 0.05$).

Table 6. Effects of different treatments of speedweed extract on its inhibition of germination of *Alternaria brassicicola* conidia

Treatment	pH value ^z	Germination (%) ^y			
		0	1,500	2,000	2,500
Extract without treatment	4.6	...	0	0	0
Cation extract resins	6.9	...	0	15	97
Anion extract resins	7.2	...	0	0	0
Activated charcoal	5.9	...	97	97	99
Water (control)	4.0	98	...	...	...

^y Percent germination of the spore suspension mixed with the same amount of diluted extract and incubated at 24°C for 8 h at 0-, 1,500-, 2,000-, and 2,500-fold dilutions.

^z pH value of the extract after treatment.

Table 7. Conidial germination of *Alternaria brassicicola* in speedweed extracts after treatment with dialysis membranes

Molecular weight minimums	Germination (%) ^z			
	0	1,500	2,000	2,500
10,000	...	96	96	94
1,000	...	97	97	97
500	...	0	0	0
100	...	0	0	0
Nontreated extract	...	0	0	0
Sterilized water	97	...	...	...

^z Spore suspension mixed with the same amount of extract was incubated at 24°C for 8 h and percent germination was determined for 0-, 1,500-, 2,000-, and 2,500-fold dilutions.

weed also contains substances strongly inhibitory to spore germination of *A. brassicicola* and the substances are very effective in controlling black leaf spot of spoon cabbage caused by this pathogen. The pH of the extract from speedweed was 4.6. Its inhibitory effect was not due to low pH because conidial germination of *A. brassicicola* was unaffected by a pH of water at 4.0. Moreover, after treatment with anion exchange resins, the pH of the extract became 7.2 and the extract was still inhibitory to spore germination.

Leaves of speedweed turn yellow and fall off in autumn, and the young twigs die during the winter. The plants begin to produce new shoots from old stems in the spring. Therefore, it was necessary to dry stems and leaves for storage and to obtain the extract from dried tissues by extraction with solvent. Results from this study showed that water, acetone, ether, or chloroform was not effective in extracting the inhibitory substances. These inhibitory substances were extracted effectively with ethanol or methanol. The inhibitory effect was not affected by treatment with anion exchange resins. However, part of the inhibitory effect of the extract disappeared and part of it remained unaffected after treatment with cation exchange resins. Results indicate that one inhibitory substance was positively charged and removable by a cation exchange resin but not by the anion resins, whereas another was uncharged and unaffected by cation or anion exchange resins. Molecular weight estimation with membrane suggests that both inhibitors have molecular weights between 500 and 1,000.

Plants tested in this study are used as medicines among oriental people (2,9). Our results show the possibility of using extracts from some of these plants to develop environmentally friendly methods for control of plant diseases.

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