Effect of Chinese Herb Extracts on Spore Germination of *Oidium murrayae* and Nature of Inhibitory Substance from Chinese Rhubarb

Y. L. Chu and **W. C. Ho,** Department of Plant Protection, National Pingtung University of Science and Technology, Nepu, Pingtung, Taiwan; and **W. H. Ko,** Department of Plant Pathology, National Chung Hsing University, Taichung, Taiwan

ABSTRACT

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More than 21% of the Chinese herbs tested contained substances in their aqueous extracts inhibitory to conidial germination of the powdery mildew fungus *Oidium murrayae*. Extracts from Chinese rhubarb and Japanese knotweed were very effective in controlling powdery mildew on cucumber, pumpkin, and eggplant. The inhibitory substance in Chinese rhubarb was soluble in polar solvents and less soluble in nonpolar solvents. The inhibitor in the aqueous extract was not dialyzable in the membrane tubing with molecular weight cut-off of 14,000, but was exchangeable by anion but not cation exchange resins, indicating that the inhibitor has a molecular weight larger than 14,000 and negative charge on its molecule.

Additional keywords: *Polygonum cuspidatum, Rheum officinale*

Powdery mildews affect many crops and represent a major global disease problem. In Western Europe, the largest amount of fungicides used is for the control of powdery mildews (5). With the high concern for adverse effects of pesticides on human health and the environment, development of alternative methods for disease control is needed. Sodium carbonate (baking soda) and plant oils emulsified with the same amount of soft soap were shown to be fungicidal against powdery mildew in the early 1930s (15–17). However, these methods remained essentially unnoticed until recent years, when interest in using bicarbonates (7,8,21) and plant oils (13,18,19) was revived.

Powdery mildews consist of a group of fungi that are extremely diverse and intricate in form and shape of reproduction structures, host range, and geographical distribution. Therefore, different methods are needed for effective control of such diverse fungi (3). A number of Chinese herbs have been used in treating infections by microorganisms (4,9). It was considered possible that some of these herbs might be useful for formulating safer products for control of powdery mildews. In this study, we screened extracts of 85 Chinese herbs for inhibition of conidial germination of powdery mildew. The best two herbs selected were tested for their ability to control powdery mildews on different hosts, and one of these two herbs was used to study the inhibition characteristics. *Oidium murrayae* Hosag. & Br. on jasmine orange (*Murraya paniculata* Jack.) was used as the test organism because of its high germination rate (more than 80% in water) in the preliminary tests. The other powdery mildew fungi tested included *Podosphaera xanthii* Br. & Shish., *Golovinomyces cucurbitacearum* (Zheng & Chen) Vakal. & Klir., *O. ipomoea* Braun, *Erysiphe polygoni* DC., *E. heraclei* Scheich. ex DC., and *Phyllactinia moricola* (Henn.) Hom., which germinated at 10 to 36% in water.

Corresponding author: W. H. Ko
E-mail: kowh@dragon.nchu.edu.tw

*The *e*-Xtra logo stands for "electronic extra" and indicates the HTML abstract available on-line contains a supplemental table of Chinese herbs not included in the print edition.

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MATERIALS AND METHODS

Source of spores. Twigs of jasmine orange with leaves covered with powdery mildew caused by *O. murrayae* were collected from the campus of National Pingtung University of Science and Technology. Conidia on the infected leaves were used to inoculate seedlings of jasmine orange in the screenhouse by gently tapping the branch held above the seedlings. Newly produced conidia on inoculated leaves were used in this study.

Preparation of herb extracts. Eighty-five Chinese herbs were purchased from Chuen Ho Tang Chinese Medicine Store, Taitung, Taiwan; these consisted of dried bark, seed, flowers, fruit, rhizomes, roots, stems, or whole plants. Three grams of each herb was crushed in 30 ml of distilled water in a 250-ml flask, autoclaved for 20 min, and filtered through a Whatman no. 1 filter paper. The filtrate, adjusted to 60 ml with distilled water, was considered a 20-fold dilution aqueous extract. For screening tests, the extracts were used without further dilution; whereas, for other tests, various dilutions of the extracts were used. Filtration and dilution of the extracts were performed under aseptic conditions.

Germination tests. To screen herb extracts for inhibition, 50 µl of extract was pipetted onto each cavity of sterile double-cavity slides containing conidia of *O. murrayae* which were added by gently tapping diseased leaves of jasmine orange held with a forceps approximately 30 cm above the slides. 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. For determining the inhibition characteristics of the Chinese rhubarb extract, glass slides were coated with cellulose acetate film by spreading 60 µl of 1% cellulose acetate in acetone (20) over a 25-by-25-mm area at the center of a slide and evaporating the acetone in a fume hood. The film area was covered with a 190-µl of extract dried in a hood, conidia of *O. murrayae* were added as described above, and slides were kept moist. Germination was recorded after incubation at 24ºC for 8 h, and 100 spores were counted in each of three replicates. Experiments were done at least twice.

Control of powdery mildews with selected herb extracts. Seed of cucumber (*Cucumis sativus* L.) cv. Wange, pumpkin (*Cucurbita moschata* Duch.) cv. Toy, and eggplant (*Solanum melongena* L.) cv. Pingtung Changche were purchased from Known-You Seed Co., Kaohsiung, Taiwan. They were grown in 10-cm pots containing a mixture of peat moss and vermiculite (9:1, vol/vol). Then, 4- to 6-week-old plants, each with two to three fully expanded leaves, were sprayed to runoff with Chinese rhubarb (*Rheum officinale* Baill.) extract or Japanese knotweed (*Polygonum cuspidatum* Sieb. & Zucc.) extract at 1,000-fold dilution sprayed every 7 days. For comparison, plants similarly were sprayed with 0.5% emulsified sunflower oil (13) or kresoxim-methyl, a new fungicide inhibitory to powdery mildews (6), at 2,000-fold dilution, as suggested by the

manufacturer (Taiwan BASF Co., Taipei, Taiwan). Control plants were sprayed with distilled water. Test plants were placed on a bench in the screenhouse to receive inocula of powdery mildew from nature. Estimation of leaf area covered with powdery mildew was made 7 days after the third spray. Twelve plants, 40 to 50 cm in height, were used for each treatment and all experiments were performed twice. Tukey's significant difference test was used to analyze the data obtained in this study.

Extraction with different solvents. Extraction was carried out by shaking 2 g of Chinese rhubarb with 20 ml of water, methanol, 50% ethanol, 95% ethanol, acetone, ethyl acetate, ether, or chloroform in a shaker at 200 strokes/min for 24 h. After filtration, the filtrate was adjusted to 20 ml with the same solvent. Then, 2 ml of the extract was evaporated to dryness in the fume hood, and the residue was redissolved in 2 ml of 50% ethanol. The solution was further diluted 40-fold with distilled water before being used for germination tests on cellulose acetate-coated slides, as described above. Ethanol, similarly diluted with distilled water, was used as the control. The experiments were performed twice.

Characterization of inhibitor in Chinese rhubarb extract. To study the effects of different treatments on the inhibitory property of the aqueous extract of Chinese rhubarb, 10 g each of Dowex anion exchange resins (Dow Chemical Co., Midland, MI), Lowatit cation exchange resins (Bayer Corporation, Pittsburgh, PA), and active charcoal (Taipei Chemical Industry Co., Hsinchu, Taiwan) was washed with 100 ml of distilled water three times by shaking over a 6-h period to remove possible inhibitory substances (11). The aqueous extract (50 ml) at 10-fold dilution was shaken with 5 g of anion exchange resins, cation exchange resins, or active charcoal in a 150-ml flask at 200 strokes/min for 24 h and filtered through a Whatman no. 1 filter paper. The filtrate was further diluted 40-fold with sterile distilled water for bioassay on cellulose acetate-coated slides.

To determine whether the inhibitory substance in the Chinese rhubarb extract is dialyzable, 2 ml of the aqueous extract at 10-fold dilution in a molecular porous membrane tubing with molecular weight cut-off of 14,000 (Spectrum Medical Industries Inc., Los Angeles, CA) was dialyzed at 24ºC against 1,000 ml of distilled water changed three times over a 24-h period.

RESULTS

Effect of aqueous extracts of Chinese herbs on spore germination. Among the 85 aqueous extracts from different Chinese herbs tested, 18 contained substances inhibitory to conidial germination of *O. murrayae*, reducing the germination rate from 86% in the water control to less than 28% in the extracts (Table 1). In all, 34 extracts were moderately inhibitory, reducing the germination rate to 30 to 69%, and 33 extracts were innocuous to *O. murrayae*, supporting 70 to 89% germination. The extracts from nine Chinese herbs which showed stronger inhibitory effect against powdery mildew fungus were selected for further tests. Aqueous extracts from Chinese rhubarb and Japanese knotweed still were strongly inhibitory to conidial germination of *O. murrayae* at 4,000-fold dilution, supporting only 5 and 11% germination, respectively (Table 2). Extracts from *P. multiflorum* Thunb., goldthread (*Coptis chinensis* Franch.), and cinnamon (*Cinnamomum cassia* Blume) showed inhibitory effect at 1,000-, 500- and 100- fold dilution, respectively, but not at further dilution. At 100-fold dilution, extracts from clove (*Eugenia caryophyllata* Thunb.) and phellodendron (*Phellodendron amurense* Rupr.) became moderately inhibitory, whereas those from ligusticum (*Ligusticum sinense* Oliver) and skullcap (*Skutellaria baicalensis* George) became only slightly inhibitory (Table 2).

Control of powdery mildews with Chinese herb extracts. Aqueous extracts of Chinese rhubarb and Japanese knotweed at 1,000-fold dilution were used to test their ability to control powdery mildews on cucumber and pumpkin caused by *Podosphaera xanthii* and on eggplant caused by *Golovinomyces cucurbitacearum* in the screenhouse. The aqueous extracts of both Chinese rhubarb and Japanese knotweed were very effective against powdery mildews on cucumber, pumpkin, and eggplant, reducing the diseased area on leaves from 24 to 55% in the control to 2 to 9% (Table 3). They were as effective as emulsified sunflower oil. Kresoxim-methyl also was very effective in reducing the severity of eggplant powdery mildew, but was only moderately effective against powdery mildew of cucumber and pumpkin (Table 3). Because of such encouraging results, Chinese rhubarb was selected for studying the nature of the substance inhibitory to powdery mildew fungi.

Nature of the inhibitory substance. The substance inhibitory to conidial germination of *O. murrayae* was extractable

Table 1. Aqueous extracts of 18 Chinese herbs inhibitory to germination of conidia of *Oidium murrayae*

Scientific name (common name)	Herb name in Chinese pronunciation	Germination (%)[z]
1. *Coptis chinensis* (goldthread)	Huang Lien	2
2. *Polygonum cuspidatum* (Japanese knotweed)	Hu Chang	3
3. *Cinnamomum cassia* (cinnamon)	Rou Gui	4
4. *Rheum officinale* (Chinese rhubarb)	Ta Huang	4
5. *Polygonum multiflorum*	Ho Shou Wu	5
6. *Eugenia caryophyllata* (clove)	Ting Shiang	8
7. *Ligusticum sinense* (ligusticum)	Chuan Shiong	8
8. *Phellodendron amurense* (phellodendron)	Huang Po	10
9. *Scutellaria baicalensis* (skullcap)	Huang chin	14
10. *Quisqualis indica* (rangoon creeper)	Su Chun Tsu	18
11. *Paeonia suffruticosa* (peony)	Mu Dan Pi	21
12. *Dictamnus dasycarpus* (Chinese dittany)	Pai Shien Pi	22
13. *Magnolia officinalis* (magnolia)	Hou Po	24
14. *Rubia cordifolia* (Indian madder)	Chian Chao Gen	24
15. *Angelica dahurica* (angelica)	Pai Tsu	26
16. *Bletilla striata* (bletilla)	Pai Ji	27
17. *Stemona sessilifolia* (stemona)	Pai Pu	27
18. *Cassia tora* (sicklepod)	Jue Ming Tsu	28
19. Water (control)	…	86

[z] Three replicates were used for each herb and 100 spores were counted for each replicate. Percentage of germination was based on the average of two tests.

Table 2. Effect of aqueous extracts of nine Chinese herbs from first selection on germination of conidia of *Oidium murrayae* at various dilutions

	Germination (%) at *n*-fold dilution[z]				
Source of extract	100	500	1,000	2,000	4,000
Chinese rhubarb	3	3	5	8	5
Japanese knotweed	2	5	8	10	11
Polygonum multiflorum	1	1	6	45	NT
Goldthread	3	9	21	33	NT
Cinnamon	8	69	NT	NT	NT
Clove	39	81	NT	NT	NT
Phellodendron	37	48	NT	NT	NT
Ligusticum	54	NT	NT	NT	NT
Skullcap	61	NT	NT	NT	NT
Water (control)	90	89	83	83	76

[z] Germinations were tested on double cavity slides; NT = not tested.

Table 3. Comparison of aqueous extracts of Chinese rhubarb and Japanese knotweed with sunflower oil and kresoxim-methyl for effectiveness in control of powdery mildew of cucumber, pumpkin, and eggplant

Treatment	Leaf area covered with powdery mildew (%)[z]		
	Cucumber	Pumpkin	Eggplant
Chinese rhubarb extract	5 A	6 A	2 A
Japanese knotweed extract	9 A	8 A	4 A
Sunflower oil	8 A	5 A	3 A
Kresoxim-methyl	41 B	25 B	5 A
Water (control)	55 C	45 C	24 B

[z] Data were taken 7 days after the third spray. Values in the same column followed by the same letter are not significantly different by Tukey's significant difference test ($\alpha = 0.05$).

Table 5. Effects of different treatment of aqueous extract of Chinese rhubarb on its inhibitory activity against germination of conidia of *Oidium murrayae*[y]

Treatment	pH value of extract after treatment	Germination (%)[z]
No treatment	4.3	0.6
Dialysis	4.5	1
Cation exchange resins	7.6	9
Anion exchange resins	6.0	83
Activated charcoal	8.0	93
Water (control)	5.9	92

[y] Aqueous extract diluted 400-fold was used in these tests.
[z] Germinations were tested on slides coated with cellulose acetate.

from Chinese rhubarb with water, methanol, 50 or 95% ethanol, acetone, or ethyl acetate. However, ether and chloroform were less effective in extracting the inhibitor (Table 4). The aqueous extract was no longer inhibitory to conidial germination after treatment with anion exchange resins or activated charcoal; however, the inhibitory effect in the extracts was not affected after dialysis or treatment with cation exchange resins (Table 5). In a separate experiment, the inhibitory effect of the extract also was not affected by raising the pH from the original 4.3 to 6.0 (*data not shown*). Dialysis treatment did not substantially affect the pH of the extract. However, the treatments with cation exchange resins, anion exchange resins, and activated charcoal increased the pH (Table 5).

DISCUSSION

More than 21% of the 85 Chinese herbs tested in this study contained substances inhibitory to the powdery mildew fungus *O. murrayae* in their aqueous extracts. Although most of the extracts were only moderately or slightly inhibitory, the amount of the fungitoxic substances extracted may be substantially increased when different extraction methods are used (12). Two herbs, Chinese rhubarb and Japanese knotweed, produced extracts that were highly effective in controlling powdery mildew on three vegetable crops.

Dried rhizomes of Chinese rhubarb are used as a medicine for lowering blood pressure and blood cholesterol levels, and as a digestive tonic. It also displays antibacterial, anthelmintic, and anticancer properties (4,9,14). Our results show that Chinese rhubarb also contains a substance inhibitory to conidial germination of *O. murrayae*. The pH of the aqueous extract from Chinese rhubarb was 4.3. Its inhibitory effect was not affected by raising the pH to 6.0. After treatment with cation exchange resins, the pH of the extract became 7.6 and the extract was still inhibitory to conidial germination of *O. murrayae*. The results indicated that the inhibition of the powdery mildew fungus by Chinese rhubarb extract was not due to low pH. The inhibitory substance in the Chinese rhubarb was extracted effectively with water, methanol, ethanol, acetone, or ethyl acetate, but less so with ether or chloroform, indicating that the inhibitor is soluble in polar solvents and less soluble in nonpolar solvents. The inhibitory substance in the aqueous extract was not dialyzable in the membrane tubing with molecular weight cut-off of 14,000, but was exchangeable by anion but not cation exchange resins, indicating that the inhibitor has a molecule weight larger than 14,000 and negative charge on its molecule. The molecular characteristics are compatible with the finding that the inhibitor is soluble in polar solvents and less soluble in nonpolar solvents. *Rheum emodi* Wall also has been reported to contain substances moderately inhibitory to fungi (1,2,10). These antifungal substances have molecular weights ranging from 250 to 550 and, therefore, are different from the inhibitory substance in Chinese rhubarb.

Table 4. Effectiveness of different solvents in extracting the substance from Chinese rhubarb inhibitory to germination of conidia of *Oidium murrayae*[z]

Solvent	Germination (%)
Water	0.3
Methanol	0.3
Ethanol (50%)	0.6
Ethanol (90%)	0.6
Ethyl acetate	0.9
Acetone	1.2
Ether	4
Chloroform	21
Control (1.25% ethanol)	92

[z] Extraction was performed by shaking 2 g of Chinese rhubarb with 20 ml of solvent for 24 h. The extract was evaporated to dryness and the residue was redissolved in 50% ethanol and diluted 400-fold with distilled water before being used to test spore germination on slides coated with cellulose acetate.

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