Distribution of Baseline Sensitivities to Natural Product Physcion Among Isolates of *Sphaerotheca fuliginea* and *Pseudoperonospora cubensis*

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ABSTRACT

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The ethanol extract from rhubarb was commercialized in China (Veguard, Physcion, 5 g/liter AS) for control of cucumber powdery mildew (*Sphaerotheca fuliginea*) and cucumber downy mildew (*Pseudoperonospora cubensis*). To provide the basic data for the risk assessment of resistance to this product, physcion was selected to represent the active ingredients to establish baseline sensitivity of powdery mildew and downy mildew populations. For powdery mildew, 262 isolates of *S. fuliginea* from nine regions and, for downy mildew populations, 116 isolates of *P. cubensis* from six regions were collected in China during 2004 and 2005 and tested for sensitivity. In addition, the sensitivity of a powdery mildew isolate was monitored for 15 asexual generations under selection pressure with physcion. The results showed that there was no significant difference among regions in the frequency distribution of baseline sensitivity to this ingredient for either cucumber powdery mildew isolates or cucumber downy mildew isolates. Baseline sensitivity was distributed as a normal unimodal curve with a mean median (50%) effective concentration (EC_{50}) of 0.304 $\mu\text{g/ml}$ for powdery mildew population and mean EC_{50} of 0.501 $\mu\text{g/ml}$ for downy mildew population. The variation of sensitivity to physcion was low because the range factor (maximum EC_{50} /minimum EC_{50} of isolates within population) varied from 1.63 to 3.42 among powdery mildew populations and from 1.70 to 2.38 among downy mildew populations. The powdery mildew isolate XZ4 did not decrease sensitivity under the selection pressure of physcion at the dose of EC_{70} for 15 generations.

Both powdery mildew, caused by *Sphaerotheca fuliginea* (Schltld.) Pollacci, and downy mildew, caused by *Pseudoperonospora cubensis* (Berk. & M.A. Curtis) Rostovzev, are widespread diseases of greenhouse and field-grown cucumber plants and can lead to significant yield loss (27,28). Despite efforts of plant breeders to develop resistant cultivars, it is not anticipated that genetic resistance will eliminate the need for fungicides (24). However, application of synthetic fungicides for plant disease control has resulted in environmental pollution, phytotoxicity to the host, and resistance of plant pathogens to fungicides (13,23,29).

To reduce reliance on synthetic fungicides, one alternative measure may be the use of fungicidal plant extracts (7,8,18). In addition to their bioactivity against a range of plant pathogens, plant extracts are biodegradable to nontoxic products and po-

tentially suitable for use in integrated management programs, and could lead to the development of a new class of disease control agents (2). The extract from leaves of the giant knotweed (*Reynoutria sachalinensis* (F. Schmidt) Nakai), containing anthraquinone physcion and so on, was commercialized as a wettable powder by Compo GmbH (Münster, Germany) in 1990. This product protects greenhouse-grown cucumber, tomato, and begonia from powdery mildew (8,12,19,20) and is also effective against *Botrytis cinerea* Pers. ex Fr. as well as *Tobacco mosaic virus* (9).

The ethanol-based extracts from the root of rhubarb (including *Rheum officinale* Baill., *R. palmatum* L., *R. tanguticum* Maxim Regel, *R. emodi* Wall., *R. crispus* L., *R. laciniatum* Prain, and others) have been shown to protect cucumber, wheat, and barley from powdery mildew fungi in greenhouse and field conditions (7,30,35–37). The extracts of rhubarb are also effective against a number of other plant pathogens, such as potato late blight (*Phytophthora infestans* (Mont.) de Bary) (15), tomato gray mold (*B. cinerea*), wheat leaf rust (*Puccinia recondita* f. sp. *tritici*), rice blast (*Magnaporthe grisea* (T.T. Hebert) M.E. Barr) (7), strawberry root rot (*Rhizoctonia solani* Kühn) (14), pepper

mosaic disease, and tomato mosaic disease (10,38).

Many studies have isolated anthraquinones from the roots of *Rheum emodi*, the seed of *Cassia tora* L., and the leaves of *R. sachalinensis* by bioassay-directed fractionation and demonstrated them to be active against a range of plant pathogens with potential to control plant diseases (7,12,18,19,36). The anthraquinones, including physcion, emodin, chrysophanol, and rhein, can be extracted using chromatography or by alkali-based methods (5,7,18,25).

Genetic resistance of plant pathogens to fungicides is a severe problem with many of the site-specific fungicides used to control crop diseases. For example, the newly developed strobilurin fungicides are used to control pathogens resistant to fungicides such as ergosterol biosynthesis inhibitors and demethylation inhibitors (22). However, the resistance to strobilurin fungicides emerged soon after they were introduced (3,13,22). The establishment of baseline sensitivity of a target plant pathogen to a new fungicide class is essential to assess the risk of fungicide resistance (16).

The ethanol plant extracts from the roots of rhubarb was commercialized for control of cucumber powdery and downy mildew (Veguard, Physcion, 5 g/liter AS; Beijing Kingbo Biotech. Co. Ltd., P.R. China) in 2007. The product contains physcion and other anthraquinone analogs, including chrysophanol, emodin, rhein, and so on. The activity of physcion against wheat powdery mildew was higher than that of other anthraquinones, and there is synergistic interaction between physcion and chrysophanol against powdery mildew pathogens (34,35). Based on its commercial potential, this compound was selected to quantify the baseline sensitivity of powdery and downy mildew populations during 2004 and 2005. A second objective was to test the likelihood of fungicide resistance by measuring the change in sensitivity of one isolate of *S. fuliginea* under selection pressure of physcion under laboratory conditions.

MATERIALS AND METHODS

Compounds used. Physcion (98%) (Fig. 1) was isolated using a modification of Chen's method (5). Ground rhizomes of

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R. officinale, purchased from the Beijing Tongrentang Co. Ltd. (Beijing) were refluxed with a solution of 20% aqueous H₂SO₄ + chloroform (1 + 5 by volume) in a water bath for 3 h. The chloroform phase was extracted with 0.25% aqueous potassium hydroxide; then, the organic solvent layer was extracted with 5% aqueous potassium hydroxide. This second aqueous fraction was acidified with hydrochloric acid to yield a yellow precipitate. The precipitate was filtered, washed with distilled water, and dried, yielding a mixture of chrysophanol and physcion. The physcion was then isolated and purified using column chromatography and identified by mass spectroscopy analysis (17). The compound was dissolved in acetone (10 mg per 10 ml) as stock solution and stored at 4°C in the dark before application.

Sample collection and isolate maintenance. During the cucumber growing season, leaves infected by powdery mildew or downy mildew were sampled from locations in nine regions, including Beijing, Wuchang, Zhengzhou, Nanjing, Tianjing, Xiaogan, Chengdu, Jingzhou, and Changsha in China during 2004 and 2005. The infected leaves were sampled from greenhouses or fields where physcion had never been applied. Leaves were kept in individual plastic bags with wet cotton covering the leafstalk for transport to the laboratory.

The powdery mildew samples were held in a chamber at 25 ± 1°C with fluorescent light at 12-h intervals for 2 days to induce conidial production. Conidia from a single colony were transferred to healthy cucum-

ber cotyledons with sterilized cotton swabs. The inoculated cotyledons were placed in covered petri dishes containing 2% water agar and kept in the chamber for propagation.

The downy mildew samples were placed in a humid chamber at 18 ± 1°C overnight in darkness to induce sporulation. The zoospores of *Pseudoperonospora cubensis* were collected from a single lesion and rinsed with sterilized distilled water using a pipette. A zoospore suspension was inoculated onto the underside of a detached, healthy cucumber leaf. The inoculated leaves were transferred to covered petri dishes containing 2% water agar and then kept in the humid chamber for propagation.

Inoculum preparation. Seven days following inoculation with powdery mildew, conidia suspensions (about 2 × 10⁵ spores/ml of distilled water, quantified by a hemacytometer) of each isolate of *S. fuliginea* were prepared by rinsing the infected leaves with sterilized distilled water containing Tween 80 (0.025%, vol/vol) with a pipette; then, each conidia suspension was passed through two layers of glass paper. Likewise, the zoospore suspensions (about 2 × 10⁴ zoospores/ml) of candidate isolate of *P. cubensis* were prepared in the same way.

Baseline sensitivity assays. A series of physcion concentrations at 10.0, 2.5, 0.63, 0.15, and 0.04 µg/ml were used for the cucumber powdery mildew assay and 12.5, 3.125, 0.78, 0.19, and 0.048 µg/ml for the cucumber downy mildew assay. Tween 80 was added in the final dilutions at the rate

of 0.025% (vol/vol). Based on the preliminary experiments, there was no difference between untreated control and negative control in disease severity. Therefore, only the negative control was utilized for data analysis.

For the powdery mildew assay, physcion was sprayed on cucumber plants at the cotyledon stage using an autosprayer tower with 0.25 MPa (Model PWT-510, manufactured by the Pesticide Machinery Group of the China Agricultural University). Spray volume was 25 ml/m². After air drying, the cotyledons were detached and put into petri dishes (60 mm in diameter) containing water agar (2%) with three cotyledons in each dish. Then the treated cotyledons were inoculated by uniformly misting leaf surfaces with conidia suspensions. After inoculation, the petri dishes were air dried to remove water drops on the leaves and then incubated in a chamber at 25 ± 1°C without light for 16 h. Spores on the surface of cotyledons were sampled with transparent tape and then the tape was placed on a slide. After adding 0.1 ml of aqueous lactic acid and glycerin (lactic acid/glycerin/water = 1/2/1, vol/vol/vol), the total conidia and germinated conidia were counted with a microscope at ×100 magnification. Germination inhibition rates (IR) were calculated as follows: IR (%) = [(GR untreated – GR treated)/GR untreated] × 100, where GR indicates germination rate.

For downy mildew assay, a leaf disc assay was used. Cucumber leaf discs (15 mm in diameter) from the fourth true leaves at the five-leaf stage were submerged in corresponding physcion solutions for 5 s and put into petri dishes (90 mm in diameter) with water agar (2%). Each petri dish had 18 leaf discs. After air drying, a zoospore suspension of 10 µl was inoculated on each leaf disc with a pipette. The petri dishes were covered and placed in a humid chamber at 18 ± 1°C in darkness overnight, then moved into a growth chamber with 18 ± 1°C and fluorescent light at 12-h intervals for disease development. Disease severities were scored 7 days after inoculation by visually assessing the percentages of leaf areas infected, and converted to % disease control according to Abbott's

Fig. 1. Chemical structure of physcion tested.

Table 1. Sensitivity to natural product physcion of *Sphaerotheca fuliginea* isolates collected from nine regions in China during 2004 and 2005

Region	No. of isolates tested	EC ₅₀ (µg/ml) ^a			RF ^b	Mean rank (<i>P</i> = 0.50) ^c
		Median	Minimum	Maximum		
Beijing	19	0.302	0.175	0.406	2.32	150.0
Wuchang	57	0.299	0.265	0.454	1.71	126.0
Jingzhou	55	0.282	0.204	0.446	2.19	117.8
Tianjing	11	0.281	0.232	0.379	1.63	122.3
Nanjing	15	0.310	0.253	0.433	1.71	152.8
Xiaogan	29	0.297	0.153	0.447	3.42	140.8
Chengdu	29	0.293	0.199	0.417	2.10	121.6
Zhengzhou	31	0.295	0.166	0.400	2.41	135.4
Changsha	16	0.312	0.217	0.364	1.68	156.3

^a EC₅₀ = mean 50% effective concentration.

^b Range factor (RF) = maximum EC₅₀/minimum EC₅₀ of isolates within population.

^c Indicates that the medians among regions are not significantly different using the Kruskal-Wallis nonparametric test.

method (1): percent disease control = $100 \times [(\text{untreated} - \text{treated})/\text{untreated}]$.

The median (50%) effective concentration (EC_{50}) for each isolate was calculated using simple linear regression of the Probit-transformed percentage of germination inhibition (for powdery mildew) or percentage of disease control (for downy mildew) against the log of physcion concentration. For the isolates of each region over year, curve shape was determined using the Kolmogorov-Smirnov test. The differences of mean ranks were determined using the Kruskal-Wallis nonparametric test. The baseline sensitivity was calculated over region and year because there were no differences either among regions or between years based on the Kruskal-Wallis nonparametric test. All analyses of data were done by SPSS 10.0.

Sensitivity monitoring of *S. fuliginea* isolate under selection pressure of physcion. Single-colony isolate XZ4 of *S. fuliginea*, with an EC_{50} value close to the sensitivity baseline, was used as the initial generation (G0). The isolate was inoculated onto cucumber cotyledons treated with physcion using a dose of 0.829 $\mu\text{g/ml}$ (about EC_{70} based on preliminary experiments). The surviving conidia were used as the first generation (G1) to inoculate cotyledons treated with physcion at the same

dose to obtain the second generation (G2). This selection was repeated for 15 generations (G15). For each generation, EC_{50} values were assessed as in the above method.

The EC_{50} value of G1 to G15 was compared with that of G0 by *t* test, and a correlation analysis was conducted to determine if the EC_{50} value changed across generations under constant selection pressure of physcion.

RESULTS

Baseline sensitivity of cucumber powdery mildew isolates. The median, maximum, and minimum EC_{50} ; population size; range factor; and mean rank of powdery mildew population from each sampling region over year are listed in Table 1. The Kruskal-Wallis nonparametric test showed that there were no significant differences in the mean rank and the distribution of baseline sensitivity among nine regions ($P = 0.505$, $P > 0.05$). The distribution given by the 262 isolates was a normal unimodal curve by Kolmogorov-Smirnov nonparametric test ($P = 0.513$, $P > 0.05$). The EC_{50} values ranged from 0.153 to 0.454 $\mu\text{g/ml}$ with a mean value $0.304 \pm 0.006 \mu\text{g/ml}$ (Fig. 2). The sensitivity range factors of populations from nine regions varied from 1.63 to 3.42 (Table 1). These results indi-

cate that there was no resistant subpopulation among the isolates of cucumber powdery mildew used in this study.

Baseline sensitivity of cucumber downy mildew isolates. Similar results were obtained using a cucumber downy mildew population. The Kruskal-Wallis nonparametric test showed that there were no significant differences in mean rank and sensitivity distribution among 6 regions ($P = 0.367$, $P > 0.05$) (Table 2). The distribution of the 116 isolates also was a normal unimodal curve ($P = 0.596$, $P > 0.05$) and the baseline sensitivity ranged from 0.280 to 0.748 $\mu\text{g/ml}$, with a mean value of $0.501 \pm 0.017 \mu\text{g/ml}$ (Fig. 3). The sensitivity range factors from the six regions were from 1.70 to 2.38 (Table 2). The finding showed that no resistant subpopulation existed among the isolates of cucumber downy mildew used in the experiment.

Sensitivity monitoring of *S. fuliginea* isolate under selective pressure of physcion. The EC_{50} values G1 to G15 of *S. fuliginea* under the selection pressure of physcion ranged from 0.249 to 0.395 $\mu\text{g/ml}$. The EC_{50} value of the initial was 0.301 $\mu\text{g/ml}$, and the *t* test showed that the EC_{50} values of G1 to G15 were not significantly different from the EC_{50} of their initial isolate. EC_{50} values of G0 to G15 did not increase with increase in generation (Fig. 4). This finding indicated the sensitivity of *S. fuliginea* remained unchanged under the selection pressure of physcion for 15 generations.

DISCUSSION

In this study, we determined the baseline sensitivities of two cucumber pathogens to physcion, a novel, natural product isolated from rhubarb plants. Powdery mildew and downy mildew were studied and established a sensitivity baseline of $0.304 \pm 0.006 \mu\text{g/ml}$ for *S. fuliginea* and $0.501 \pm 0.017 \mu\text{g/ml}$ for *P. cubensis*. These baselines will be used to monitor any shift of sensitivity in cucumber powdery mildew and downy mildew populations to physcion.

The variations in the powdery mildew and downy mildew populations to physcion were relatively low. In the selection assay, it appeared that sensitivity of *S. fuliginea* was stable after 15 generations of continuous selection. In the case of stro-

Fig. 2. Baseline sensitivity of *Sphaerotheca fuliginea* isolates (262 isolates from 2004 and 2005) to physcion collected from nine regions in China. Mean median (50%) effective concentration (EC_{50}) value = $0.304 \pm 0.006 \mu\text{g/ml}$.

Table 2. Sensitivity to natural product physcion of *Pseudoperonospora cubensis* isolates collected from six regions in China during 2004 and 2005

Region	No. of isolates tested	EC_{50} ($\mu\text{g/ml}$) ^a			RF ^b	Mean rank ($P = 0.50$) ^c
		Median	Minimum	Maximum		
Beijing	17	0.498	0.325	0.748	2.30	60.0
Wuchang	32	0.443	0.280	0.667	2.38	49.4
Jingzhou	14	0.521	0.418	0.709	1.70	67.6
Nanjing	15	0.472	0.310	0.730	2.35	52.3
Xiaogan	21	0.514	0.401	0.689	1.72	67.1
Zhengzhou	17	0.502	0.360	0.710	1.97	61.5

^a EC_{50} = 50% effective concentration.

^b Range factor (RF) = maximum EC_{50} /minimum EC_{50} of isolates within population.

^c Indicates that the medians among regions is not significantly different using the Kruskal-Wallis nonparametric test.

bilirin fungicides, changes in sensitivity could be found within one to three growing seasons (31), which is equal to approximately 5 to 15 generations of *S. fuliginea*. Han et al. (11) reported that the range in the baseline sensitivity of cucumber downy mildew to azoxystrobin, one of the strobilurin fungicides, was 79.8 (minimum 11.7×10^{-5} µg/ml and maximum 9.34×10^{-3} µg/ml). This indicates that the variation of sensitivity to this type of fungicide was very high in the pathogen population compared with our findings with physcion. Strobilurin fungicides are site-specific inhibitors which act as inhibitors of electron transport from cytochrome b to c1 on the mitochondrial respiration system and, therefore, are considered to have a high potential resistance risk for resistance development in pathogen populations when they are used in agricultural settings (32).

Plant extracts of *R. officinale* have been used in traditional Chinese herbal medicine and are widely used in clinics to treat the hepatitis B virus (26,33). Studies have shown that the mechanism of action of the anthraquinones, including emodin, rhein, aloe-emodin physcion, and chrysophanol, for treating the human pathogen *Staphylococcus aureus* was inhibition of protein synthesis (21). Studies have investigated activity against the carcinoma cells of *Ehrlich ascites* through inhibition of respiration, oxidation, and dehydrogenization of some amino acids and intermediate products of carbohydrates metabolism and glycolysis (6), and as inhibitors of some enzymes of the mitochondrial electron transport system (4). However, the action mechanism of anthraquinones against plant pathogens has not been determined.

The results of this study show little variation in sensitivity among the populations of the two distinct plant pathogens to the fungicide physcion. Both plant pathogens have high reproduction rates and, as such, are at higher risk for resistance development. After 15 generations of exposure, there was no change in EC₅₀. Based on this evidence, it is likely that physcion will have a low to moderate risk for resistance development.

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Fig. 3. Baseline sensitivity of *Pseudoperonospora cubensis* isolates (116 isolates from 2004 and 2005) to physcion collected from six regions in China. Mean median (50%) effective concentration (EC₅₀) value = 0.501 ± 0.017 µg/ml.

Fig. 4. Sensitivity dynamics of *Sphaerotheca fuliginea* under the selection pressure of physcion. Median (50%) effective concentration (EC₅₀) values of *S. fuliginea* to physcion ranged from 0.249 to 0.395 µg/ml and were not significantly different from the initial isolate XZ4 by *t* test. The correlation analysis ($R^2 = 0.027$) indicated the EC₅₀ values did not increase with the increase of generation under the selection of the compound.

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