Baseline Sensitivity of Pyraclostrobin and Toxicity of SHAM to *Sclerotinia sclerotiorum*

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Abstract

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Sclerotinia sclerotiorum is a cosmopolitan plant pathogen notable for its wide host range. The quinone outside inhibitor (QoI) fungicide pyraclostrobin has not been registered for control of *S. sclerotiorum* in China. In this study, baseline sensitivity of pyraclostrobin was established based on effective concentration for 50% inhibition of mycelial growth (EC_{50}) values of 153 isolates of *S. sclerotiorum* collected from five provinces of China and toxicity of alternative oxidase inhibitor salicylhydroxamic acid (SHAM) to *S. sclerotiorum* was determined. Results showed that the frequency distribution of EC_{50} values of the 153 isolates was unimodal but with a right-hand tail. The mean EC_{50} value was 0.1027 $\mu\text{g/ml}$ and the range of EC_{50} values was 0.0124 to 0.6324 $\mu\text{g/ml}$. Applied as a preventive fungicide in pot experiments,

pyraclostrobin at 5, 15, and 45 $\mu\text{g/ml}$ provided control efficacies of 61, 77, and 100%, respectively. There was no positive cross-resistance between pyraclostrobin and carbendazim or dimethachlon. EC_{50} values for SHAM against four isolates of *S. sclerotiorum* were 44.4, 51.8, 54.4, and 68.7 $\mu\text{g/ml}$. SHAM at 20 $\mu\text{g/ml}$ could significantly increase not only the inhibitory effect of pyraclostrobin on mycelial growth on potato dextrose agar media but also the control efficacy in planta. These results indicated that SHAM should not be added into artificial media in in vitro assay of *S. sclerotiorum* sensitivity to pyraclostrobin. This has broad implications for assay of sensitivity of fungal pathogen to QoI fungicides.

The ascomycete fungus *Sclerotinia sclerotiorum* (Lib.) de Bary is a highly destructive plant pathogen capable of infecting more than 400 species of plants worldwide, including economically important crops and vegetables such as oilseed rape, soybean, sunflower, peanut, cabbage, cauliflower, and tomato (4,5). It was reported that the annual losses caused by *S. sclerotiorum* in the United States exceeded U.S.\$200 million in the late 20th century (5). In Jiangsu Province of eastern China, yield losses of oilseed rape caused by this pathogen range from 10 to 80% (33). Diseases caused by *S. sclerotiorum* have traditionally been difficult to control because resistant cultivars have not yet been sufficiently developed in major crops. In practice, application of fungicides is still the principal method for control of *S. sclerotiorum* in oilseed rape in China (33). The benzimidazole fungicide carbendazim was extensively used to control *S. sclerotiorum* during the 1980s and 1990s in China. As a result, high levels of resistance to carbendazim were reported in the late 1990s and became widespread in eastern China in the following years (22,29,39). Mainly due to field control failures caused by the development of resistance to carbendazim, the dicarboximide fungicides dimethachlon, iprodione, and procymidone began to be commonly used for control of *S. sclerotiorum* around the 2000s in China. More than a decade of dimethachlon applications resulted in low levels of resistance to dimethachlon in Jiangsu Province of eastern China (19). More recently, medium to high levels of resistance to dimethachlon have been reported in Shaanxi Province of northwestern China, Hunan Province of central China, and Heilongjiang Province of northeastern China. Furthermore, there was positive cross-resistance among different members of the dicarboximide fungicide group (40,41). With the development of resistance to traditionally used fungicides, it is important and urgent to find

proper substitute fungicides for successful management of *S. sclerotiorum*.

The quinone outside inhibitor (QoI) fungicides are the most important class of agricultural fungicides in respect to market share and sales. The discovery of this class of fungicides was based on a group of natural products synthesized by wood-rotting fungi, including one called *Strobilurus tenacellus*. Hence, the QoI fungicides are also referred to as the strobilurins in recognition of the source of the lead compounds for development of this class of fungicides (31). The biochemical mode of action of QoI fungicides is to block electron transport at the quinol-oxidizing site of the cytochrome bcl complex (complex III) in the mitochondrial respiration chain, thus halting synthesis of adenosine triphosphate. The QoI fungicides are effective in inhibiting sporulation, spore germination, and mycelial growth of the target fungal pathogens. Because of their broad-spectrum activity, QoI fungicides have been widely used in agriculture (3,34). Pyraclostrobin belongs to the QoI class and was developed and commercialized by BASF Corporation (Ludwigshafen, Germany) in the early 2000s. It was reported that pyraclostrobin has excellent preventive, curative, and eradication activities against a wide range of plant pathogens (38). Thus far, pyraclostrobin has not yet been registered in China on oilseed rape or soybean for control of *S. sclerotiorum*. The only registered QoI fungicide for control of *S. sclerotiorum* in China was tricyclopiricarb (China Pesticide Information Network, <http://www.chinapesticide.gov.cn>). At present, no case of *S. sclerotiorum* resistance to QoI fungicide has been reported in China.

Establishment of baseline sensitivity before introduction of a new fungicide into market is essential for subsequent resistance monitoring programs and is required during the registration process in the European Union (27). It has been reported that fungi may avoid the fungitoxic effects of QoI fungicides through the alternative oxidase (AOX) located on the mitochondrial membrane whereas, during fungal infection of plants, AOX is presumed to be inhibited by plant secondary metabolites such as flavonoid (13,35). Therefore, an AOX characteristic inhibitor, salicylhydroxamic acid (SHAM), is commonly included in artificial culture media to suppress the alternative respiration (9,20,21). In most cases, the addition of SHAM, usually at 100 $\mu\text{g/ml}$, will reduce effective concen-

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tration for 50% inhibition of mycelial growth (EC_{50}) values for the tested QoI fungicide, as demonstrated in *Ascochyta rabiei* (34) and *S. sclerotiorum* (9). However, the dose response to QoI fungicide in the presence of SHAM may not always represent the real sensitivity to the tested fungicide for some fungal plant pathogens. For example, EC_{50} value for the QoI fungicide azoxystrobin in the presence of SHAM against *Fusicladium effusum* was higher, rather than lower, than that for azoxystrobin alone (28). The EC_{50} values of the sensitive isolates of *Microdochium nivale* were also higher for azoxystrobin in the presence of SHAM (0.5 mM) compared with azoxystrobin without SHAM (32). The sole purpose for addition of SHAM into artificial media during in vitro assay of QoI fungicide sensitivity is to inhibit AOX. However, these results indicate that the inhibition of AOX is not the only effect of SHAM on plant pathogens; thus, the EC_{50} values of QoI fungicide in the presence of SHAM may overestimate or underestimate the real QoI fungicide sensitivity. This is consistent with the report that the biological activity of SHAM on plant pathogens is pathogen dependent (35). Whether SHAM should be added into artificial media in in vitro assay of pyraclostrobin sensitivity in *S. sclerotiorum* deserves an investigation. Therefore, the objectives of this study were to (i) establish the baseline sensitivity of *S. sclerotiorum* to pyraclostrobin, (ii) assess preventive and curative activities of pyraclostrobin against *S. sclerotiorum*, and (iii) investigate the toxic effect and role of SHAM in assay of *S. sclerotiorum* sensitivity to pyraclostrobin.

Materials and Methods

Origins and collections of *S. sclerotiorum* isolates. Isolates ($N = 153$) of *S. sclerotiorum* were collected in 2013 from the following hosts and regions: 62 isolates from oilseed rape fields in Hubei and Hunan Provinces of central China, 62 isolates from oilseed rape fields in Gansu and Shaanxi Provinces of northwestern China, and 29 isolates from soybean fields in Heilongjiang Province of northeastern China. The collection fields were separated from one another by more than 20 km. In each field, several plants with typical symptoms of Sclerotinia stem rot were arbitrarily selected. Sclerotia collected from the same infected stem of oilseed rape or soybean plant were regarded as one isolate.

Fungicides. All fungicides were technical-grade products. Pyraclostrobin (95% active ingredient [a.i.]) was provided by Hubei Kang Bao Tai Fine-Chemical Co. Ltd. and dissolved in acetone to produce a 1,000- μ g/ml stock solution. Dimethachlon (96.2% a.i.) was provided by Wenzhou Pesticide Co. Ltd. and dissolved in methanol to produce a 1,000- μ g/ml stock solution. Carbendazim (98.1% a.i.) was provided by Haili Guixi Chemical Co. Ltd. and dissolved in hydrochloric acid (HCl) at 0.1 mole/liter to produce a 1,000 μ g/ml stock solution. The stock solutions were stored in a refrigerator at 4°C for no longer than 2 weeks before being serially diluted for bioassay experiments.

Determination of EC_{50} values and baseline sensitivity of *S. sclerotiorum* to pyraclostrobin. Determination of the EC_{50} was performed according to Ma et al. (19), with minor modifications. Briefly, potato dextrose agar (PDA) media were routinely amended with pyraclostrobin at final concentrations of 0.0125, 0.025, 0.05, 0.1, 0.2, and 0.4 μ g a.i./ml. For some isolates, one or two extra concentrations of 0.003125, 0.00625, or 0.8 μ g a.i./ml were used to ensure that, for each isolate, there was at least one concentration of pyraclostrobin having a mycelial growth percent inhibition greater than 50% and, at the same time, at least another concentration having a percent inhibition lower than 50%. Mycelial plugs (6 mm in diameter) cut from the periphery of 2-day-old colonies of each isolate were placed upside down onto the center of fungicide-amended PDA plates. The fungicide-free PDA plates were used as the control. PDA plates were incubated at 26°C for 48 h in the dark. The diameter of each colony was measured twice at right angles. The experiments were performed in triplicate. Pyraclostrobin concentrations and their respective percent inhibitions were used to calculate EC_{50} values by the procedure of Bioassay Analysis for Quantitative Data in Data Processing System (ver.

7.05; Hangzhou RuiFeng Information Technology Co. Ltd.). Baseline sensitivity of pyraclostrobin was constructed based on frequency distributions of EC_{50} values of the 153 isolates.

Determination of cross-resistance between pyraclostrobin and carbendazim or dimethachlon. Three dimethachlon-resistant isolates from Heilongjiang Province, three carbendazim-resistant isolates from Hunan Province, and three dual-sensitive isolates (sensitive to both dimethachlon and carbendazim) from Hunan, Gansu, and Heilongjiang Provinces were used to determine the pattern of cross-resistance among pyraclostrobin, dimethachlon, and carbendazim. EC_{50} values of the nine isolates for the three fungicides were assayed as described above. Serial concentrations of dimethachlon and carbendazim were prepared so that, for each isolate, there was at least one concentration with a percent inhibition above 50% and at least another concentration with a percent inhibition below 50%. Correlation coefficients (r) between sensitivity to pyraclostrobin and that to dimethachlon and carbendazim were calculated by the correlation procedure in GraphPad Prism (version 5.01; GraphPad Software, Inc.) from the logarithmically transformed EC_{50} values.

Preventive and curative activities of pyraclostrobin against *S. sclerotiorum* on detached leaves of oilseed rape. Leaves of similar size (12 to 15 cm in diameter) were detached from 7- to 8-week-old oilseed rape plants, rinsed with sterile water, air dried, and transferred to 15-cm petri dishes lined with wet filter paper to maintain high humidity. Pyraclostrobin solutions were prepared by diluting pyraclostrobin stock solution with 0.1% Triton X-100 in water. Detached leaves were sprayed with pyraclostrobin solutions at 10, 20, and 40 μ g/ml by a hand-held sprayer (Xinmeir Co. Ltd.). Carbendazim was used as the reference fungicide. The treated leaves were air dried for 1 h, then inoculated on the adaxial surface with inverted mycelial plugs cut from actively growing margins of 2-day-old colonies on PDA media. The inoculated leaves were incubated in a growth chamber at 26°C. Lesion diameters were measured twice at right angles after 24 and 48 h of incubation. For determination of curative activity, the leaves were inoculated with mycelial plugs and incubated for 24 h, then sprayed with fungicide dilutions as described above. Lesion diameters were measured twice at right angles 24 and 48 h after fungicide application. The experiments were performed in quadruplicate.

Preventive and curative activities of pyraclostrobin against *S. sclerotiorum* in pot experiments. Preventive and curative activities of pyraclostrobin on potted oilseed rape plants were assessed with a similar procedure as described above. Seven- to eight-week-old oilseed rape plants growing in 20-cm-diameter pots containing a composite mixture of peat and soil (weight ratio, 1:1) in the greenhouse at $26 \pm 1^\circ\text{C}$ were used to substitute for detached leaves. The concentrations of pyraclostrobin and carbendazim were 5, 15, and 45 μ g/ml with the 0.1% Triton X-100 in water as the control. Oilseed rape plants were sprayed with fungicide solutions until runoff. Lesion diameters were measured twice at right angles after 48 and 72 h of incubation for preventive activity, and 48 and 72 h after fungicide application for curative activity. The experiments were arranged in a completely randomized design with four replicate oilseed rape plants for each treatment.

Toxic effects of SHAM on mycelial growth of *S. sclerotiorum* on PDA. Technical-grade product of SHAM (99% a.i.; Sigma-Aldrich) was dissolved and serially diluted with methanol to obtain final concentrations of 10, 20, 40, 80, 160, and 320 μ g/ml in PDA media. PDA amended with only methanol at a final concentration of 0.2% (vol/vol) was used as the control. Mycelial plugs (6 mm in diameter) cut from the periphery of 2-day-old colonies of 10 isolates with pyraclostrobin EC_{50} values lower than 0.1 μ g/ml were placed upside down onto the center of SHAM-amended PDA plates. PDA plates were incubated at 26°C for 48 h in the dark. The diameter of each colony was measured twice at right angles. The experiments were performed in triplicate.

Similarly, in determination of EC_{50} values for pyraclostrobin in the presence of SHAM, the stock solution of SHAM was added to a duplicate set of pyraclostrobin-amended PDA to provide a final

concentration of SHAM at 20 µg/ml. PDA amended with methanol at a final concentration of 0.2% (vol/vol) was used as the control. Measurements of mycelial growth and subsequent calculations of EC₅₀ values were the same as described above for pyraclostrobin without SHAM.

Potential of control efficacy of pyraclostrobin by SHAM in pot experiments. The experiment procedure was similar to that for determination of preventive control efficacy of pyraclostrobin in pot experiments, as described above. Stock solution of SHAM (dissolved in methanol at 10,000 µg/ml) was diluted with 0.1% Triton X-100 in water to get a final concentration of 20 µg/ml. The mixture solution of pyraclostrobin and SHAM (at final concentrations of 10 and 20 µg/ml, respectively) was prepared accordingly. Control efficacies of SHAM at 20 µg/ml, pyraclostrobin at 10 µg/ml, and a mixture of pyraclostrobin and SHAM after 72 h of incubation were calculated as described above. Co-toxicity factor was calculated according to Kosman and Cohen (16) with the formula co-toxicity factor = (control efficacy of mixture – theoretical control efficacy of mixture)/theoretical control efficacy of mixture, where theoretical control efficacy of mixture = control efficacy of SHAM + control efficacy of pyraclostrobin – control efficacy of SHAM × control efficacy of pyraclostrobin.

Statistical analysis. Control efficacies were calculated by the following formula. The diameter of mycelial plugs was subtracted from the measured lesion diameters before calculating control efficacies: control efficacy (%) = [(lesion diameter of the control – lesion diameter of the treated)/lesion diameter of the control] × 100.

The procedure of Tukey's multiple comparison test of analysis of variance in GraphPad Prism (version 5.01; GraphPad Software, Inc.) was used to test significant differences in the mean EC₅₀ values between different *S. sclerotiorum* populations. Student's *t* test was employed to compare control efficacy under the same conditions between carbendazim and pyraclostrobin. Control efficacy data were arcsine square-root transformed before statistical analysis. EC₅₀ data were logarithmically transformed for analysis of correlation between sensitivity to different fungicides.

Results

Baseline sensitivity of *S. sclerotiorum* to pyraclostrobin. The mean EC₅₀ values for the five populations of *S. sclerotiorum* collected from different provinces of China were very similar to one another. There was no significant difference ($P = 0.9758$) in mean EC₅₀ values among the five populations (Table 1). Therefore, individual EC₅₀ values for the 153 isolates were pooled together to construct a combined sensitivity baseline for isolates from different regions of China. The frequency distribution of the EC₅₀ values was a unimodal curve, albeit with a long right-hand tail (Fig. 1). The mean EC₅₀ value for the 153 isolates of *S. sclerotiorum* was 0.1027 µg/ml and the range of EC₅₀ values was 0.0124 to 0.6324 µg/ml (Table 1).

Cross-resistance between pyraclostrobin and carbendazim, dimethachlon. The correlation coefficient (*r*) between EC₅₀ values for pyraclostrobin and those for dimethachlon was 0.282 while $r =$

–0.476 for pyraclostrobin and carbendazim (Fig. 2). None of the correlations was statistically significant ($P = 0.462$ and 0.195 , respectively) and, therefore, no positive or negative cross-resistance between pyraclostrobin and either dimethachlon or carbendazim was detected.

Preventive and curative activities of pyraclostrobin against *S. sclerotiorum* on detached leaves of oilseed rape. Experiments on detached leaves showed that pyraclostrobin provided higher preventive control efficacies than reference fungicide carbendazim, and the curative control efficacies for the two fungicides were lower than their respective preventive control efficacies (Table 2). Pyraclostrobin applied at 10 µg/ml 1 h before inoculation provided 93% reduction in disease severity whereas preventive control efficacy of carbendazim at the same concentration was only 45%. In regards to curative efficacy, pyraclostrobin applied at 10 µg/ml 24 h after inoculation exhibited fungicidal activities similar to those of carbendazim.

Preventive and curative activities of pyraclostrobin against *S. sclerotiorum* in pot experiments. Control efficacies on potted oilseed rape plants showed that preventive fungicidal activities of pyraclostrobin at 5 and 45 µg/ml 72 h after inoculation were significantly higher ($P < 0.05$) than those of carbendazim (Table 3). However, at a concentration of 15 µg/ml, the difference was not significant ($P > 0.05$) between the two fungicides. At all three concentrations, the curative activities of pyraclostrobin were similar to those of carbendazim. Compared with preventive activities, the curative activities of pyraclostrobin were significantly lower ($P < 0.05$). Pyraclostrobin at 45 µg/ml provided only 50% curative control efficacy whereas the preventive control efficacy at the same concentration was 100%.

Toxic effects of SHAM on mycelial growth of *S. sclerotiorum* on PDA media. EC₅₀ values for SHAM against four *S. sclerotio-*

Fig. 1. Frequency distribution of pyraclostrobin sensitivity (effective concentration for 50% inhibition of mycelial growth [EC₅₀] values) of 153 isolates of *Sclerotinia sclerotiorum* collected from different regions of China. Individual EC₅₀ values were grouped in class intervals of 0.04 µg/ml. Values on the X-axis indicate the midpoint of the interval.

Table 1. Pyraclostrobin sensitivity in five populations of *Sclerotinia sclerotiorum* isolates collected from different regions of China

Host	Collection region	N ^y	EC ₅₀ (µg/ml) ^x		
			Range	Mean ± standard deviation	Significance ^z
Oilseed rape	Hubei	32	0.0249–0.3090	0.1110 ± 0.0660	a
	Hunan	30	0.0252–0.6324	0.1048 ± 0.1268	a
	Gansu	30	0.0336–0.2861	0.1009 ± 0.0506	a
	Shaanxi	32	0.0156–0.5138	0.1005 ± 0.1297	a
Soybean	Heilongjiang	29	0.0124–0.1973	0.0955 ± 0.0511	a
Total	...	153	0.0124–0.6324	0.1027 ± 0.0916	...

^x Determination of effective concentration for 50% inhibition of mycelial growth (EC₅₀) values was based on inhibition of mycelial radial growth on PDA media.

^y Number of isolates.

^z Tukey's multiple comparison test of analysis of variance procedure in GraphPad Prism (ver. 5.01) at $P = 0.05$ was employed for multiple comparisons between different populations of isolates. The same letter in the column indicates there was no significant difference in the mean EC₅₀ values at 5% significance level.

orum isolates (HN26, HB17, HB4, and GS10) were 44.40, 51.80, 54.43, and 68.68 µg/ml, respectively. No inhibitory effects on mycelial growth on PDA media were observed for SHAM at a final concentration of 10 µg/ml (Fig. 3). SHAM at 40 µg/ml could significantly inhibit mycelial growths of all four isolates. These results indicated that SHAM at a concentration equal to or greater than 20 µg/ml had apparent toxic effects on mycelial growth of *S. sclerotiorum* on PDA media.

Potential of pyraclostrobin in vitro toxicity and control efficacy by SHAM. EC₅₀ values for pyraclostrobin were greatly reduced by addition of SHAM into PDA media. Of the 10 tested isolates, individual EC₅₀ values of 7 isolates for pyraclostrobin with SHAM at 20 µg/ml were significantly lower than their respective EC₅₀ values for pyraclostrobin without SHAM (Table 4). The average EC₅₀ value of the 10 isolates for pyraclostrobin without SHAM was 0.0587 µg/ml whereas the average EC₅₀ value for pyraclostrobin with SHAM was 0.0173 µg/ml. Taking the 10 isolates as a group, a paired *t* test showed that the difference in the mean EC₅₀ values was extremely significant (*P* < 0.0001) between pyra-

clostrobin with and without SHAM. Similarly, SHAM at a concentration of 20 µg/ml greatly increased control efficacies of pyraclostrobin against three of the four tested isolates in pot experiments. The co-toxicity factors for SHAM and pyraclostrobin against isolate HN18, HN26, and SX23 were higher than 67 (Table 5). However, for control of isolate HB4, antagonism was detected between SHAM and pyraclostrobin.

Discussion

Baseline sensitivity data are valuable for resistance risk assessment. The wide range of the baseline EC₅₀ values for pyraclostrobin and especially the long right-hand tail on the sensitivity distribution curve (Fig. 1) may imply a high risk for resistance development in the future although, at present, no definite correlation can be established between the range of sensitivity distribution and the risk of resistance development (7). The site-specific mode of action of pyraclostrobin also indicates a high potential for development of resistance, as do QoI fungicides in general. In most cases, only one point mutation in the mitochondrial *cytochrome b*

Fig. 2. Pearson correlation coefficients (*r*) between sensitivity to pyraclostrobin and sensitivity to A, dimethachlon and B, carbendazim.

Table 2. Preventive and curative activity of pyraclostrobin against *Sclerotinia sclerotiorum* on detached leaves of oilseed rape^x

Conc (µg/ml) ^z	Preventive control efficacy (%)				Curative control efficacy (%) ^y			
	24 h after inoculation		48 h after inoculation		24 h after application		48 h after application	
	Pyra	Carb	Pyra	Carb	Pyra	Carb	Pyra	Carb
10	100.00	49.99*	92.59	44.95*	19.39	20.54	31.54	28.83
20	100.00	56.97*	100.00	52.36*	58.07	23.74*	74.44	39.05*
40	100.00	82.14	100.00	69.55*	66.91	44.79*	77.31	63.83

^x Pyra = pyraclostrobin, Carb = carbendazim, and * indicates the control efficacy of carbendazim was significantly different from that of pyraclostrobin under the same conditions according to Student's test at *P* = 0.05. Control efficacy data were transformed by arcsine square root method before statistical analysis.

^y For curative control efficacy, fungicides were applied 24 h after inoculation.

^z Concentration.

Table 3. Preventive and curative activity of pyraclostrobin against *Sclerotinia sclerotiorum* in pot experiments^x

Conc (µg/ml) ^z	Preventive control efficacy (%)				Curative control efficacy ^y			
	48 h after inoculation		72 h after inoculation		48 h after application		72 h after application	
	Pyra	Carb	Pyra	Carb	Pyra	Carb	Pyra	Carb
5	79.76	57.94*	61.25	42.70*	29.79	37.97	18.39	17.34
15	100.00	76.19*	76.67	62.92	44.04	45.74	32.12	26.60
45	100.00	100.00	100.00	74.90*	80.99	63.64	50.05	57.96

^x Pyra = pyraclostrobin, Carb = carbendazim, and * indicates the control efficacy of carbendazim was significantly different from that of pyraclostrobin under the same conditions according to Student's test at *P* = 0.05. Control efficacy data were transformed by arcsine square root method before statistical analysis.

^y For curative control efficacy, fungicides were applied 24 h after inoculation.

^z Concentration.

gene leading to an amino-acid change from glycine to alanine at position 143 (G143A) may confer high levels of resistance to QoI fungicide (34). The Fungicide Resistance Action Committee (FRAC) categorized QoI fungicides as high risk for resistance development (10). QoI fungicides were first commercially available in 1996 and within, 2 to 4 years of commercial marketing, resistance was detected in field populations of several pathogen species (11). Thus far, there have been numerous reports of QoI fungicide resistance in a variety of fungal plant pathogens, including *Alternaria solani* (23,24), *Botrytis cinerea* (12,15), *Cercospora beticola* (Sacc.) (20), *Erysiphe graminis* f. sp. *tritici* (8), *Magnaporthe grisea* (1), *M. oryzae* (18), *Pyricularia grisea* (14), and *Ustilago maydis* (42).

Fig. 3. Mycelial growth inhibitions of *Sclerotinia sclerotiorum* by increasing concentrations of salicylhydroxamic acid (SHAM) on potato dextrose agar media. The error bar indicates standard deviation (SD) of three replicates. Calculated SHAM effective concentration for 50% inhibition of mycelial growth values for isolates HN26, HB17, HB4, and GS10 were 44.40, 51.80, 54.43, and 68.68 µg/ml, respectively.

Pyraclostrobin has been reported to be an excellent systemic fungicide with both preventive and curative activities on a wide range of fungal plant pathogens (38). The present study demonstrated that the preventive control efficacy was higher than that of carbendazim but the curative control efficacy of pyraclostrobin applied 24 h after inoculation was similar to that of carbendazim. The spray volume in this study was higher than commonly applied in the field, so that every leaf could be sprayed until runoff. However, the concentrations used in this study were much lower than the recommended label rate for pyraclostrobin against other pathogens. In the case of *Sphaerotheca cucurbitae* on cucumber, the recommended label rate in China is about 80 to 250 µg/ml. Because the biochemical mode of action of pyraclostrobin is different from those of carbendazim and dimethachlon, there is no positive cross-resistance between pyraclostrobin and either carbendazim or dimethachlon, as demonstrated in this study. Therefore, pyraclostrobin should be an excellent fungicide candidate for management of *Sclerotinia sclerotiorum* resistant to carbendazim or dimethachlon. Nevertheless, great care should be taken and precautions must be adopted to avoid or delay the development of resistance to pyraclostrobin because *S. sclerotiorum* has shown a high potential for resistance development to commonly used fungicides such as dimethachlon (19,40,41) and, especially, carbendazim (17,25,39). According to the guidelines recommended by FRAC for management of fungicide resistance, judicious fungicide application strategies include (i) not using a single fungicide exclusively, (ii) restricting the number of applications per season, and (iii) applying fungicides as preventative protectants rather than as eradicants (6). Integrated pathogen management tactics such as disease-resistant plant varieties, biological control agents, crop rotations, and mixtures or rotations of pyraclostrobin with other fungicides with different modes of action should be implemented to delay or prevent the possible development of pyraclostrobin resistance in *S. sclerotiorum*.

The common practice of adding SHAM to artificial media in assay of QoI fungicide sensitivity is for inhibition of AOX, which is

Table 4. *Sclerotinia sclerotiorum* effective concentration for 50% inhibition of mycelial growth (EC₅₀) values for pyraclostrobin with and without salicylhydroxamic acid (SHAM)

Isolate	Pyraclostrobin with SHAM ^w		Pyraclostrobin without SHAM		Significance ^y
	EC ₅₀ (µg/ml)	95% CI of EC ₅₀ ^x	EC ₅₀ (µg/ml)	95% CI of EC ₅₀ ^x	
HN1	0.0156	0.0107–0.0226	0.0632	0.0462–0.0865	*
HN26	0.0175	0.0053–0.0580	0.0993	0.0637–0.1548	*
HB4	0.0253	0.0183–0.0351	0.0680	0.0527–0.0877	*
HB17	0.0166	0.0059–0.0467	0.0524	0.0316–0.0869	...
GS22	0.0140	0.0092–0.0215	0.0465	0.0419–0.0517	*
GS10	0.0216	0.0107–0.0435	0.0438	0.0307–0.0624	...
SX5	0.0134	0.0111–0.0163	0.0362	0.0347–0.0378	*
SX23	0.0145	0.0062–0.0340	0.0380	0.0279–0.0518	...
HLJ23	0.0126	0.0073–0.0217	0.0646	0.0392–0.1065	*
HLJ13	0.0216	0.0116–0.0403	0.0754	0.0506–0.1126	*
Average	0.0173	...	0.0587	...	<i>P</i> < 0.0001 ^z

^w Final concentration of SHAM in potato dextrose agar media was 20 µg/ml.

^x The 95% confidence interval (CI) was calculated by statistical software package Data Processing System (ver. 7.05; Hangzhou RuiFeng Information Technology Co. Ltd.).

^y An asterisk (*) denotes that difference in EC₅₀ values between pyraclostrobin with and without SHAM was statistically significant. The criterion for significant difference in EC₅₀ values was according to Robertson and Preisler (26), as follows: the two EC₅₀ values were considered significantly different if there was no overlap for 95% CIs of the two EC₅₀ values.

^z Student's paired *t* test was employed for significant difference in the mean EC₅₀ values of the 10 isolates between pyraclostrobin with and without SHAM.

Table 5. Potentiation of control efficacy of pyraclostrobin against *Sclerotinia sclerotiorum* in pot experiments by SHAM

Isolate	Control efficacy (%)			Co-toxicity factor ^z
	SHAM (20 µg/ml)	Pyraclostrobin (10 µg/ml)	SHAM + Pyraclostrobin (20 + 10 µg/ml)	
SX23	9.32	42.78	81.28	68.94
HN26	–9.14	45.97	82.90	102.04
HN18	8.95	46.33	85.52	67.25
HB4	7.62	62.11	47.19	–27.40

^z Co-toxicity factor greater than 20 denotes synergism and co-toxicity factor lower than –20 denotes antagonism.

presumed to be inhibited during infections by plant secondary metabolites such as flavonoid (35). If fungal AOX can be completely inhibited in planta, then it is incomprehensible that SHAM can significantly increase control efficacies of pyraclostrobin, as has been shown in the present study. One possible explanation is that the secondary metabolites of oilseed rape might not, or at least not completely, inhibit AOX of *S. sclerotiorum* during infections. Up to the present, no direct biochemical evidence has been reported that AOX can be inhibited by secondary metabolites of oilseed rape during infections. Another possible reason for the significantly increased control efficacy may be the direct toxicity of SHAM on *S. sclerotiorum*. Studies by Xu et al. (37) demonstrated that AOX is involved in the regulation of growth, development, and resistance to oxidative stress of *S. sclerotiorum*. Nonetheless, the direct toxic effects of SHAM at 20 µg/ml or higher concentrations on *S. sclerotiorum* may not be caused totally by the inhibition of AOX because AOX is only the branched oxidase to the core respiratory pathway (35). The toxic effect of SHAM may be partially due to its inhibitory activity on other essential fungal enzymes, such as peroxidase, putative melanogenesis enzymes, esterase, and laccase (2,30). The toxic effect of SHAM has been reported on other fungal pathogens; for example, even at low concentrations, SHAM could suppress mycelial growth of *F. effusum* on PDA media (28). With respect to the direct effect of SHAM on fungicide sensitivity assays, previous studies also demonstrated that SHAM at 100 µg/ml could decrease EC₅₀ value of the QoI fungicide azoxystrobin against *S. sclerotiorum* but increase EC₅₀ value of the dicarboximide fungicide procymidone (9,36). This evidence indicates that addition of SHAM into artificial media in assay of QoI fungicide sensitivity may lead to inaccurate or erroneous results. Therefore, at least for determination of *S. sclerotiorum* sensitivity to pyraclostrobin based on mycelial growth inhibition on PDA media, SHAM should not be added.

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Literature Cited

- Avila-Adame, C., and Köller, W. 2003. Characterization of spontaneous mutants of *Magnaporthe grisea* expressing stable resistance to the Qo inhibiting fungicide azoxystrobin. *Curr. Genet.* 42:332-338.
- Baek, H. S., Rho, H. S., Yoo, J. W., Ahn, S. M., Lee, J. Y., Lee, J., Kim, M., Kim, D. H., and Chang, I. S. 2008. The inhibitory effect of new hydroxamic acid derivatives on melanogenesis. *Bull. Korean Chem. Soc.* 29:43-46.
- Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A., Hamer, M., and Parr-Dobrzanski, B. 2002. The strobilurin fungicides. *Pest Manage. Sci.* 58:649-662.
- Boland, G. J., and Hall, R. 1994. Index of plant hosts of *Sclerotinia sclerotiorum*. *Can. J. Plant Pathol.* 16:93-108.
- Bolton, D. M., Thomma, B. P., and Nelson, B. D. 2006. *Sclerotinia sclerotiorum* (Lib.) de Bary: Biology and molecular traits of a cosmopolitan pathogen. *Mol. Plant Pathol.* 7:1-16.
- Brent, K. J., and Hollomon, D. W. 2007. Fungicide resistance in crop pathogens: How can it be managed? FRAC Monograph No. 1. 2nd ed. Brussels. http://www.frac.info/publication/anhang/FRAC_Mono1_2007_100dpi.pdf
- Brent, K. J., and Hollomon, D. W. 2007. Fungicide resistance: The assessment of risk. FRAC Monograph No. 2. Second Ed. Brussels. http://www.frac.info/publication/anhang/FRAC_Mono2_2007.pdf
- Chin, K. M., Chavillaz, D., Kaesbohrer, M., Staub, T., and Felsenstein, F. G. 2001. Characterizing resistance risk of *Erysiphe graminis* f. sp. *tritici* to strobilurins. *Crop Prot.* 20:87-96.
- Duan, Y. B., Liu, S. M., Ge, C. Y., Feng, X. J., Chen, C. J., and Zhou, M. G. 2012. In vitro inhibition of *Sclerotinia sclerotiorum* by mixtures of azoxystrobin, SHAM and thiram. *Pestic. Biochem. Physiol.* 103:101-107.
- FRAC (Fungicide Resistance Action Committee). 2014. FRAC Code List 2014: Fungicide sorted by mode of action. <http://www.frac.info/publication/anhang/2014%20FRAC%20Code%20List.pdf>
- Gisi, U., Sierotzki, H., Cook, A., and McCaffery, A. 2002. Mechanisms influencing the evolution of resistance to QoI inhibitor fungicides. *Pest Manage. Sci.* 58:859-867.
- Ishii, H., Fountaine, J., Chung, W., Kansako, M., Nishimura, K., Takahashi, K., and Oshima, M. 2009. Characterization of QoI-resistant field isolates of *Botrytis cinerea* from citrus and strawberry. *Pest Manage. Sci.* 65:916-922.
- Kaneko, I., and Ishii, H. 2009. Effect of azoxystrobin on activities of anti-oxidant enzymes and alternative oxidase in wheat head blight pathogens *Fusarium graminearum* and *Microdochium nivale*. *J. Gen. Plant Pathol.* 75:388-398.
- Kim, Y. S., Dixon, E. W., Vincelli, P., and Farman, M. L. 2003. Field resistance to strobilurin (QoI) fungicides in *Pyricularia grisea* caused by mutations in the mitochondrial cytochrome b gene. *Phytopathology* 93:891-900.
- Kim, Y. K., and Xiao, C. L. 2011. Stability and fitness of pyraclostrobin- and boscalid-resistant phenotypes in field isolates of *Botrytis cinerea* from apple. *Phytopathology* 101:1385-1391.
- Kosman, E., and Cohen, Y. 1996. Procedures for calculating and differentiating synergism and antagonism in action of fungicide mixtures. *Phytopathology* 86:1263-1272.
- Kuang, J., Wang, J. X., and Zhou, M. G. 2011. Monitoring carbendazim and dimethachlon resistance of *Sclerotinia sclerotiorum* obtained from the blight stems of rape in Jiangsu Province. *Chin. Agric. Sci. Bull.* 27:285-291.
- Ma, B., and Uddin, W. 2009. Fitness and competitive ability of an azoxystrobin-resistant G143A mutant of *Magnaporthe oryzae* from perennial ryegrass. *Plant Dis.* 93:1044-1049.
- Ma, H. X., Feng, X. J., Chen, Y., Chen, C. J., and Zhou, M. G. 2009. Occurrence and characterization of dimethachlon insensitivity in *Sclerotinia sclerotiorum* in Jiangsu Province of China. *Plant Dis.* 93:36-42.
- Malandrakis, A. A., Markoglou, A. N., Nikou, D. C., Vontas, J. G., and Ziogas, B. N. 2006. Biological and molecular characterization of laboratory mutants of *Cercospora beticola* resistant to Qo inhibitors. *Eur. J. Plant Pathol.* 116:155-166.
- Markoglou, A. N., Malandrakis, A. A., Vitoratos, A. G., and Ziogas, B. N. 2006. Characterization of laboratory mutants of *Botrytis cinerea* resistant to QoI fungicides. *Eur. J. Plant Pathol.* 115:149-162.
- Pan, Y. L. 1998. The resistance of *Sclerotinia sclerotiorum* of rape to carbendazim and its management. *Jiangsu J. Agric. Sci.* 14:159-163.
- Pasche, J. S., Piche, L. M., and Gudmestad, N. C. 2005. Effect of the F129L mutation in *Alternaria solani* on fungicides affecting mitochondrial respiration. *Plant Dis.* 89:269-278.
- Pasche, J. S., Wharam, C. M., and Gudmestad, N. C. 2004. Shift in sensitivity of *Alternaria solani* in response to QoI fungicides. *Plant Dis.* 88:181-187.
- Qi, Y. X., Chen, F. X., Su, X. Y., Ding, K. J., Yu, J., and Jiang, M. S. 2006. Monitoring on carbendazim resistance of *Sclerotinia sclerotiorum* obtained from the blight stems of rape in Anhui Province. *Chin. Agric. Sci. Bull.* 22:371-373.
- Robertson, J. L., and Preisler, H. K. 1992. Pesticide Bioassays with Arthropods. CRC Press, Inc., Boca Raton, FL.
- Russell, P. E. 2004. Sensitivity baselines in fungicide resistance research and management. FRAC Monograph No. 3. Brussels. <http://www.frac.info/publication/anhang/monograph3.pdf>
- Seyran, M., Brenneman, T. B., and Stevenson, K. L. 2010. In vitro toxicity of alternative oxidase inhibitors salicylhydroxamic acid and propyl gallate on *Fusicladium effusum*. *J. Pestic. Sci.* 83:421-427.
- Shi, Z. Q., Zhou, M. G., and Ye, Z. Y. 2000. Resistance of *Sclerotinia sclerotiorum* to carbendazim and dimethachlon. *Chin. J. Oil Crop Sci.* 22:54-57.
- Tsukamoto, K., Itakura, H., Sato, K., Fukuyama, K., Miura, S., Takahashi, S., Ikezawa, H., and Hosoya, T. 1999. Binding of salicylhydroxamic acid and several aromatic donor molecules to *Arthromyces ramosus* peroxidase, investigated by x-ray crystallography, optical difference spectroscopy, NMR relaxation, molecular dynamics, and kinetics. *Biochemistry* 38:12558-12568.
- Vincelli, P. 2002. QoI (Strobilurin) fungicides: Benefits and risks. The Plant Health Instructor. doi:10.1094/PHI-I-2002-0809-02 <http://www.apsnet.org/edcenter/advanced/topics/Pages/StrobilurinFungicides.aspx>
- Walker, A., Auclair, C., Gredt, M., and Leroux, P. 2009. First occurrence of resistance to strobilurin fungicides in *Microdochium nivale* and *Microdochium majus* from French naturally infected wheat grains. *Pest Manage. Sci.* 65:906-915.
- Wang, Y., Hou, Y. P., Chen, C. J., and Zhou, M. G. 2014. Detection of resistance in *Sclerotinia sclerotiorum* to carbendazim and dimethachlon in Jiangsu Province of China. *Australas. Plant Pathol.* 43:307-312.
- Wise, K. A., Bradley, C. A., Pasche, J. S., Gudmestad, N. C., Dugan, F. M., and Chen, W. 2008. Baseline sensitivity of *Ascochyta rabiei* to azoxystrobin, pyraclostrobin, and boscalid. *Plant Dis.* 92:295-300.
- Wood, P. M., and Hollomon, D. W. 2003. A critical evaluation of the role of alternative oxidase in the performance of strobilurin and related fungicides acting at the Qo site of Complex III. *Pest Manage. Sci.* 59:499-511.
- Xu, T., Wang, Y. T., Liang, W. S., Yao, F., Li, Y. H., Li, D. R., Wang, H., and Wang, Z. Y. 2013. Involvement of alternative oxidase in the regulation of sensitivity of *Sclerotinia sclerotiorum* to the fungicides azoxystrobin and procymidone. *J. Microbiol.* 51:352-358.
- Xu, T., Yao, F., Liang, W. S., Li, Y. H., Li, D. R., Wang, H., and Wang, Z. Y. 2012. Involvement of alternative oxidase in the regulation of growth, development, and resistance to oxidative stress of *Sclerotinia sclerotiorum*. *J. Microbiol.* 50:594-602.
- Yang, L. J., and Bai, Y. L. 2012. Strobilurin fungicide—Pyraclostrobin.

39. Zhang, X. L., Sun, X. M., Zhang, G. F., Yi, H. J., and Qiang, Y. 2003. Preliminary report on the monitoring of the resistance of *Sclerotinia sclerotiorum* to carbendazim and its integrated management. Chin. J. Pestic. Sci. Adm. 24:18-22.
40. Zhou, F., Zhang, X. L., Li, J. L., and Zhu, F. X. 2014. Dimethachlon resistance in *Sclerotinia sclerotiorum* in China. Plant Dis. 98:1221-1226.
41. Zhou, F., Zhu, F. X., Zhang, X. L., and Zhang, A. S. 2014. First report of dimethachlon resistance in field isolates of *Sclerotinia sclerotiorum* on oilseed rape in Shaanxi Province of northwestern China. Plant Dis. 98:568.
42. Ziogas, B. N., Markoglou, A. N., and Tzima, A. 2002. A non-Mendelian inheritance of resistance to strobilurin fungicides in *Ustilago maydis*. Pest Manage. Sci. 58:908-916.