Baseline Sensitivity and Control Efficacy of Pyraclostrobin Against *Botryosphaeria dothidea* Isolates in China

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Abstract

Botryosphaeria dothidea is an important fungal pathogen that causes apple ring rot, which can significantly reduce apple yield. Fungicide applications are the main control measure of apple ring rot worldwide. Pyraclostrobin is a quinone outside inhibitor (QoI) fungicide that has yet to be registered for control of *B. dothidea* in China. Baseline sensitivity of *B. dothidea* to pyraclostrobin (EC₅₀ of mycelial growth inhibition) was assessed for 97 isolates collected in Shandong Province. The EC₅₀ values ranged from 0.7010 to 7.1378 µg/ml with the mean value of 3.0870 µg/ml and displayed a unimodal frequency distribution. After

cultured on fungicide-free PDA medium or on apples for multiple generations, the *B. dothidea*-resistant isolates (RS^T) remained resistant to pyraclostrobin, but exhibited similar virulence as the susceptible isolates (S^T). Cross-resistance investigation revealed that pyraclostrobin was not cross-resistant to tebuconazole, flusilazole, carbendazim, and iprodione. Field evolution showed that pyraclostrobin at 200 and 250 g a.i./ha provided greater than 80% control efficacy against apple ring rot disease when applied as a therapeutic or preventive fungicide. The efficacy was similar to fungicides that have been registered for apple.

Botryosphaeria dothidea (Moug.) Ces. & De Not causes ring rot disease that adversely affects apple yields in major apple producing regions around the world (Marsberg et al. 2017; Xu et al. 2015). Infected fruits show lesions consisting of circular rings alternating in colors of dark and light brown. The incidence of decayed fruits may reach 30 to 40% before harvest and greater than 70% in storage (Zhang et al. 2012a, 2012b). The pathogen also can cause large numbers of cankers on branches and trunks, leading to tree fatality in severe cases (Tang et al. 2012).

Cultivation practices, such as fruit bagging, removal or girdling infected branches, have been used to protect apple orchards from *B. dothidea*. However, these practices can negatively affect tree health and are labor intensive (Dai et al. 2017). Preventive application of fungicides of different chemical groups is still a practical and effective strategy for managing *B. dothidea*. There are a number of fungicides registered against apple ring rot, including benzimidazoles (carbendazim, thiophanate-methyl), sterol demethylation inhibitors (flusilazole, tebuconazole), and dithiocarbamate (mancozeb) (Fan et al. 2016a; Liu et al. 2013). Due to extensive use, *B. dothidea* resistances to tebuconazole, carbendazim, and thiophanate-methyl have already reported (Ma et al.

2000; Wang et al. 2010; Yang and Liu 2002). Therefore, identifying low-risk and more effective fungicides is urgently needed for *B. dothidea* management.

Pyraclostrobin (a QoI fungicide) has shown excellent activity against a wide range of plant fungal pathogens, such as ascomycetes, basidiomycetes, adelomycetes, and oomycetes, when applied preventively, curatively, or for eradication (Yang and Liu 2002; Yuan et al. 2015). Since commercial introduction in China in 2005, pyraclostrobin has been registered for control of banana scab, banana leaf spot, cucumber powdery or downy mildew, and northern leaf blight of corn. Unfortunately, due to the extensive application of pyraclostrobin with a single site of action and medium-to-high resistance risk (Bartlett et al. 2002; FRAC 2014; Sun et al. 2015), many plant fungal pathogens, such as *Botrytis cinerea* (Xiao et al. 2017; Yin et al. 2012), *Cercospora beticola* (Birla et al. 2012), *Cercospora soja* (Zhang et al. 2012a), and *Sclerotinia sclerotiorum* (Liang et al. 2015), have developed resistance to pyraclostrobin.

Although pyraclostrobin is not registered for control of *Botryosphaeria* ring rot on apples in China (China Pesticide Information Network: <http://www.icama.org.cn/>), it has been used in apple orchards for years. Good inhibitory effects against apple ring rot were demonstrated (Duan et al. 2012; Fan et al. 2016a), and no resistant *B. dothidea* isolate to pyraclostrobin has been reported to date. Baseline sensitivity establishment is a significant part of the registration process for all fungicides in the European Union (Di et al. 2016), and baseline data would potentially be required by the Chinese registration authority because the information is important for resistance management programs. The current study has objectives of (a) establishing baseline sensitivity of *B. dothidea* collected from Shandong Province to pyraclostrobin, (b) evaluating the pathogenicity and stability of the isolates identified with low or high pyraclostrobin sensitivity under laboratory conditions, (c) determining pyraclostrobin cross-resistance patterns with tebuconazole, flusilazole, carbendazim, and iprodione, and (d) evaluating field efficacy of pyraclostrobin against apple ring rot.

Materials and Methods

Fungicides. Technical-grade pyraclostrobin (97.5% a.i., BASF SE [China]), tebuconazole (96.0% a.i., Jiangsu Huanghai Pesticide

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Co. Ltd.), flusilazole (95.0% a.i., Shandong Weifang Rainbow Chemical Co., Ltd.), and difenoconazole (92.0% a.i., Syngenta Crop Protection [China]) were dissolved in acetone at 10,000 µg/ml as stock solutions. Technical-grade carbendazim (98.1% a.i., Tianjin Jin Bei Chemical Co. Ltd.) was dissolved in hydrochloric acid (HCl, 0.1 mol/liter) to produce a 10,000 µg/ml stock solution. These stock solutions were stored at 4°C in the dark before use. Serial dilutions were made using double-distilled water containing 0.05 µg/ml of Tween 80 (Sigma-Aldrich, St Louis, MO) within 2 weeks of experiments.

Fungal isolates and identification. Apple fruits with ring rot symptoms were collected from 18 apple orchards in Shandong Province of China between July and August of 2015–2016 (Supplementary Table S1). The orchards are at least 40 km apart and with no history of pyraclostrobin exposure. To isolate *B. dothidea* (a total of 97 isolates), infected fruits were surface-sterilized in 0.05% sodium hypochlorite solution (10% commercial bleach solution) for 3 min, and then triple rinsed in sterilized water. A small piece of tissue was taken from the margin of a lesion and incubated with acidified Difco potato dextrose agar (PDA) in a 90-mm petri dish at 26°C for 4–5 days. Then, a single hyphal tip was collected from each colony, transferred to a fresh PDA plate, incubated at 26°C for 3 days, and then stored at 4°C for later use. Four or five typical isolates collected from each orchard were used in the study.

The isolates were initially identified as *Botryosphaeria* spp. based on colony characteristics and microscopic morphology. Their morphological characters were compared with previous descriptions of *B. dothidea* (Peng et al. 2011; Tang et al. 2012; Xu et al. 2015). The identity of all isolates in the study was further confirmed by phylogenetic analysis combined with the complete nuclear rDNA internal transcribed spacer (ITS) region and partial sequence of translation elongation factor 1 alpha (EF-1α) following the description by Tang et al. (2012) and Xu et al. (2015). Raw sequences of isolates collected in this study were obtained from Shengggong Biotech Co., Ltd. (Shanghai, P.R. China), and the other five reference isolates, including two isolates of *B. dothidea* (MAFF645001 and CMW8000),

isolates of *B. cortices* (CBS119047), *Phyllosticta citricarpa* (CBS127454), and *Neofusicoccum vitifusiforme* (STE-U5252) were downloaded from GenBank following Blast searches or references to published papers (Liu et al. 2013). All nucleotide sequences were initially aligned with the software Clustal X 2.0, and then imported into BioEdit 5.0.9.1 for optimizing manually to analyze their nucleotide polymorphisms. Phylogenetic reconstructions of concatenated and individual gene-trees were performed using Maximum parsimony (MP) and Bayesian Markov Chain Monte Carlo criteria. In addition, to determine whether the combined sequences can be used to construct phylogenetic analyses, statistical congruence was examined by applying a partition homogeneity test (PHT). PAUP 4.0b10 was used to separately analyze single genes and combined genes to construct parsimony trees. The cultural characteristics were observed on 2% PDA after 7 days at 25°C. For sporulation, the isolates cultured on 2% malt extract agar (MEA) were induced under UV light with 12/12 h light and dark cycles. Mature pycnidia of each isolate were collected from MEA and crushed in sterile-distilled water in a microcentrifuge tube. The conidial suspension was then filtered through three layers of lens paper to remove mycelia and pycnidial debris. Conidial morphology was observed with a light microscope at ×400 magnification, and the length and width of 50 conidia per isolate were determined.

In vitro bioassay of pyraclostrobin sensitivity. Pyraclostrobin sensitivity was assessed using a mycelial growth inhibition method (Zhai et al. 2014). PDA medium plates containing 12.0, 6.0, 3.0, 1.5, 0.75, or 0.375 µg/ml pyraclostrobin were prepared by mixing 49 ml medium (at ~50°C) with 1 ml pyraclostrobin dilution of various concentrations (the acetone content of the diluted solutions was maintained at 0.1%). The control medium was not amended with the fungicide but prepared by using sterile water containing the same amount of Tween 80 and acetone. Isolates were precultured on PDA plates for 4 days at 26°C in darkness. A 7-mm-diameter PDA plug with mycelia was cut from the growth edge of the fungal colony and placed face-down at the center of the previously prepared fungicide-amended or control PDA plates. After 3 days of incubation, colony diameters (radial growth) were manually measured in two perpendicular directions. The measurements were corrected by subtracting the diameter of the agar plug. For each concentration and isolate, four plates were prepared and measured. The experiments were conducted twice. The measurements were used to estimate the effective concentration for 50% inhibition of mycelial growth (EC₅₀).

Pathogenicity evaluation. From the results of the pyraclostrobin sensitivity tests, resistant isolates (RS^T) (Qixia, QX5, Yiyuan, YY10, and Feicheng, FC6, isolates with the lowest pyraclostrobin sensitivity surveyed) and susceptible isolates (S^T) (Taian, TA3, and TA9 with the highest pyraclostrobin sensitivity surveyed) were selected for pathogenicity tests on apple shoots and detached fruits in May–October of 2015 and 2016. New shoots on 2-year-old apple seedlings (*Malus domestica* ‘Fuji’) in an orchard (36°11′N, 111°08′E, Tai’an, Shandong Province) were inoculated in the field. The inoculation sites on selected shoots were first surface-sterilized by 70% ethanol. After the ethanol dried, a wound (2 mm in diameter and 2 mm in depth) was created by a disinfected nail (with 70% ethanol). A mycelial plug (5 mm diameter) cut from the growth edge of a 5-day-old culture was placed on the wound and covered with sterile and moist cotton wool wrapped with plastic wrap. After 7 days, the plastic wrap, cotton wool, and PDA plug were removed. The sites were marked for evaluation (lesion size) at 5 days after. Four inoculation sites were established on a given shoot, and there were five

Fig. 1. Frequency distribution of pyraclostrobin sensitivity of 97 *Botryosphaeria dothidea* isolates.

Table 1. Lesion diameter (cm) of RS^T and S^T isolates on untreated apple shoots and fruits^z

	Resistant isolates (RS ^T)			Sensitive isolates (S ^T)	
	QX5	YY10	FC6	TA3	TA9
On shoot	5.32 ± 0.055	5.42 ± 0.026	5.38 ± 0.039	5.51 ± 0.045	5.35 ± 0.052
On apple	5.82 ± 0.047	5.71 ± 0.040	5.75 ± 0.057	5.69 ± 0.037	5.80 ± 0.047

^z Mean ± SEM. No significant difference was found.

shoots for each isolate for a total of 20 replicates. The same number of shoots was similarly inoculated with PDA plugs without fungus as controls.

For detached apple fruits, two wounds (1.5 mm in diameter and 2 mm in depth) were created on each fruit using a sterilized nail. Four mycelial plugs (5 mm diameter) cut from the growth edge of a 5-day-old culture were placed on the surface of the wounded fruits. Control fruits were inoculated with sterile PDA plugs. All apples with inoculants were placed on trays in plastic containers with a layer of water (approximately 5 cm) at the bottom and maintained at 26°C. Ten apples (replications) were inoculated with each isolate. The experiment was performed twice. At 5 days after inoculation, the number of decayed fruits was recorded, and lesion diameters were measured.

Stability of pyraclostrobin sensitivity. The stability of pyraclostrobin sensitivity was investigated by subculturing resistant isolates (QX5, YY10, FC6) and susceptible isolates (TA3 and TA9) on fungicide-free PDA for 15 generations as well as on fungicide-free apple fruits for 4 generations. Apple fruits were inoculated using the method described above. Reisolation was performed using the decayed fruits, and the resulting mycelia were used for next-generation inoculation. Ten apples were inoculated at each generation. EC₅₀ of pyraclostrobin was determined at the seventh and 15th generations on fungicide-free PDA and for each generation inoculated on fungicide-free apple fruits. The entire test was repeated twice. The EC₅₀ values were compared with the initial EC₅₀ values to evaluate the stability of the low and high pyraclostrobin sensitivity.

Cross-resistance evaluation. Nine isolates from different apple growing regions with a range of sensitivity levels to pyraclostrobin (EC₅₀ = 0.7218, 1.3460, 2.6375, 3.1790, 3.7300, 4.3900, 5.3125, 6.2215, and 7.1378 µg/ml) were selected to assess their sensitivity to tebuconazole, flusilazole, carbendazim, and iprodione using the method described above. The final concentrations were 0, 0.5, 1.0, 5.0, 10.0, and 20.0 µg/ml for tebuconazole; 0, 0.50, 1.00, 5.00, 10.00, and 50.00 µg/ml for flusilazole; 0, 0.50, 1.00, 5.00, 10.00, and 20.00 µg/ml for difenoconazole; and 0, 0.50, 1.00, 5.00, 10.00, and 50.00 µg/ml for carbendazim. There were four replicates for each isolate and fungicide combination, and the experiment was repeated twice.

Field efficacy of pyraclostrobin. Field trials were conducted during 2015 and 2016 in two orchards naturally infested by *B. dothidea* in Qufu (35°59'N, 116°98'E) and Feicheng (36°24'N, 116°76'E), Shandong Province. The experiment contained seven treatments: 250 g/liter pyraclostrobin EC (BASF, China) at 125, 200, and 250 g a.i./ha; 43% tebuconazole SC (Bayer, China) at 107.5 g a.i./ha; 50% carbendazim WP (Xingnong, China) at 833.3 g a.i./ha; 400 g/liter flusilazole EC (Xingnong, China) at 50 g a.i./ha; and an untreated control (CK). Each plot consisted of 2 trees, and were arranged in a randomized block design with four blocks (replicates) and one tree buffer between the plots and blocks. The fungicide treatments were applied using a Jacto Heavy-Duty HD400 sprayer (Agrolex, Singapore) at 3,000 liters/ha. A total of 7 applications were made at 15-day intervals with the first application at the initiation of the

disease in early May. No other fungicides were applied to the experimental plots during the experiment. Herbicide and fertilizer applications were applied following the standard farm practices. Disease incidence was evaluated 10 days after the last spraying by counting the total number of fruits and the diseased fruits on each treated tree. After the field evaluation, a total of 50 randomly selected symptomless fruits (5 fruits at each of the five directions around a treated tree) were collected and placed in a plastic package, and stored at room temperature. Diseased fruits were counted at 15 and 30 days after storage, and percent disease incidence was calculated as: the number of diseased apples/total number of apples × 100%. Control efficacy was calculated using the abbot formula (Chen et al. 2008; Zhang et al. 2009).

Data analysis. All data were analyzed using SAS software (version 9.4, SAS Institute Inc., Cary, NC). EC₅₀ values were estimated using probit analysis. The EC₅₀ frequency distribution was plotted using PROC FREQ and tested for normality. Cross-resistance patterns were analyzed using Spearman rank correlation and line regression analysis. The virulence of resistant and susceptible isolates, and field trial data were analyzed by ANOVA followed by Tukey's multiple range tests for mean separations. Fisher's least significant difference (LSD) test ($P = 0.05$) was used to determine significant difference among EC₅₀ values, disease incidence, and control efficacy.

Results

Baseline sensitivity of *B. dothidea* to pyraclostrobin. From the 18 selected orchards, a total of 97 *B. dothidea* isolates were obtained and identified morphologically and genetically (Supplementary Fig. S1). These isolates were tested for pyraclostrobin sensitivity. The EC₅₀ values ranged from 0.7010 to 7.1378 µg/ml with the mean value of 3.0870 ± 0.1560 (SE) µg/ml. The frequency distribution of the EC₅₀ values displayed as a unimodal curve (Fig. 1) that follows a normal (Gaussian) distribution ($P = 0.200 > 0.05$, KS normality test procedure) in a symmetrical manner as indicated by a skewness value of 0.586 and a kurtosis value of 0.114.

Virulence of RS^T and S^T isolates. Both RS^T and S^T isolates were able to cause lesions on untreated shoots in the field as well as on detached apples in the laboratory with similar virulence. The pathogenicity of the RS^T and S^T isolates were similar, as there were no significant differences between isolates with regard to lesion diameter on shoots ($F = 2.5810$, $P = 0.0799$) or apples ($F = 1.346$, $P = 0.2987$). The lesion diameter ranged from 5.32 to 5.51 cm on shoots and from 5.69 to 5.82 cm on apple fruits at 5 days after inoculation (Table 1).

Sensitivity stability of isolates. After subcultured 15 generations on fungicide-free PDA and 4 generations on fungicide-free apple fruits, similar resistance factors were observed (Table 2). No significant difference was found for the EC₅₀s after subculture ($P > 0.05$, Fisher's least significant difference test), suggesting none of the resistant isolates became more sensitive after generations without selection pressure, and the inheritance of the pyraclostrobin resistance was stable (Table 2).

Table 2. Pyraclostrobin EC₅₀ (ppm) of 5 isolates after 4 consecutive generations on fungicide-free apple fruits^z

	QX5			YY10		
	EC ₅₀	95%CL	Slope	EC ₅₀	95%CL	Slope
On PDA						
Seventh generation	7.2261	6.4948–8.1409	0.9200	3.0140	2.5203–3.5434	0.9903
15th generation	7.1834	6.1760–8.4807	0.9563	3.0501	2.4836–3.7521	1.0769
On apple						
First generation	7.1378	5.9776–8.5470	1.0203	3.0600	2.6107–3.6383	1.0688
Second generation	7.2042	6.1651–8.3263	0.9306	3.0046	2.3480–3.7962	1.0689
Third generation	7.1864	6.0109–8.5681	0.9769	3.3940	2.7644–3.9457	0.9431
Fourth generation	7.1261	6.3516–7.9666	0.9696	3.1428	2.7870–3.5108	1.0256

(Continued on next page)

^z Resistant isolates: QX5, YY10, and FC6; susceptible isolates: TA3 and TA9; CL: confidence limit.

Cross-resistance patterns. The correlation coefficient (R^2) between EC_{50} values for pyraclostrobin and other tested fungicides was 0.2596 for tebuconazole, 0.1048 for flusilazole, 0.237 for carbendazim, and 0.0387 for iprodione (Fig. 2). None of the correlations was statistically significant ($P = 0.401, 0.209, 0.062$, and 0.276 , respectively) suggesting no cross-resistance between pyraclostrobin and the four tested fungicides.

Field efficacy of pyraclostrobin. Pyraclostrobin exhibited excellent activity against apple ring rot in the field trials. The disease incidence of the untreated control (CK) ranged from 19.31 to 23.35% preharvest and 58.10 to 73.35% postharvest, demonstrating high disease pressure. No significant differences were found between orchards and seasons ($P = 0.8946$ and 0.1486 for orchard and year, respectively). In 2015, pyraclostrobin at 200 and 250 g a.i./ha provided greater than 80% control efficacy, similar to registered fungicides. In 2016, pyraclostrobin at 250 g a.i./ha showed significantly higher control efficacy compared with other treatments (Table 3).

At 125 g a.i./ha, the control efficacy of pyraclostrobin was significantly lower than that of the other fungicides ($P < 0.05$). The disease control efficacies were generally lower for postharvest for all fungicide treatments since the apple fruits were stored at room temperature (Table 3).

Discussion

Establishing fungicide baseline sensitivity before commercialization is a good reference for future resistance monitoring programs if the baseline represents the natural sensitivity variation of the given pathogen (Lu et al. 2015). The present study is the first attempt to characterize pyraclostrobin sensitivity of *B. dothidea* in China. The 97 isolates of *B. dothidea* used in the study were from orchards that had never been exposed to pyraclostrobin. Therefore, the mean EC_{50} value (3.0870 ± 0.1560 $\mu\text{g/ml}$) is representative and can be a reference for future evaluation of potential shifts in pyraclostrobin sensitivity among *B. dothidea* populations in China. The range of the EC_{50} values is from 0.7010

Fig. 2. Correlation analysis of EC_{50} s between pyraclostrobin and A, tebuconazole, B, flusilazole, C, carbendazim, and D, iprodione.

Table 2. (Continued from preceding page)

FC6			TA3			TA9		
EC_{50}	95% CL	Slope	EC_{50}	95% CL	Slope	EC_{50}	95% CL	Slope
1.8920	1.6010–2.2251	1.0175	0.7966	0.6519–0.9743	1.0753	0.7211	1.0723	0.5848–0.8880
1.9241	1.5697–2.3012	1.0830	0.7836	0.6297–0.9750	1.0114	0.7302	1.0460	0.5659–0.9502
1.8510	1.5540–2.2576	1.0038	0.7860	0.6652–0.9317	1.0667	0.7010	1.0648	0.6070–0.9277
1.9382	1.7944–2.1087	0.9587	0.7937	0.6411–0.9843	1.0218	0.7861	1.0062	0.6566–0.9282
1.8216	1.5794–2.0951	1.0586	0.7821	0.6378–0.9549	1.0483	0.6824	1.0302	0.5347–0.8326
1.9015	1.6256–2.1886	1.0690	0.7855	0.6347–0.9720	1.0303	0.7420	1.0384	0.5772–0.9651

Table 3. Field efficacy (% control) against *Botryosphaeria dothidea* during 2015 and 2016^z

Orchard	Fungicide	g a.i./ha	In field		15 days postharvest		30 days postharvest	
			2015	2016	2015	2016	2015	2016
Qufu	Pyraclostrobin 250 g/liter EC	125	77.62 c	79.21 c	66.35 d	69.60 c	57.32 c	59.75 b
		200	80.35 b	82.35 b	68.28 cd	71.05 bc	60.50 b	65.30 a
		250	83.55 a	85.64 a	72.31 a	74.96 a	63.35 a	67.25 a
	Tebuconazole 43% SC	107.5	82.02 ab	84.20 ab	70.52 ab	72.52 b	61.08 ab	65.85 a
	Carbendazim 50% WP	833.3	81.37 ab	83.63 ab	69.61 bc	72.03 b	60.37 b	65.05 a
	Flusilazole 400 g/liter EC	50	82.72 ab	84.39 ab	71.32 ab	73.10 ab	61.75 ab	65.90 a
	CK-disease incident (%)		23.35	21.20	61.25	60.02	73.35	71.20
Feicheng	Pyraclostrobin 250 g/liter EC	125	78.86 c	79.86 c	67.20 c	68.92 c	58.85 c	57.35 c
		200	80.75 bc	83.62 b	69.62 bc	70.35 bc	61.32 b	61.25 b
		250	84.30 a	86.97 a	73.30 a	74.65 a	63.89 a	64.32 a
	Tebuconazole 43% SC	107.5	81.25 b	84.10 b	70.25 b	71.20 bc	61.45 b	61.80 b
	Carbendazim 50% WP	833.3	81.10 b	83.25 b	68.65 bc	70.82 bc	60.72 bc	61.05 b
	Flusilazole 400 g/liter EC	50	82.18 b	83.85 b	69.27 bc	71.55 b	61.65 b	62.30 ab
	CK-disease incident (%)		20.40	19.31	59.35	58.10	71.68	70.15

^z Means followed by the same letter within columns indicating no significant difference according to Tukey's multiple range test at $P = 0.05$.

to 7.1378 $\mu\text{g/ml}$, representing a range-of-variation factor (VF) of 10.18. Furthermore, the frequency distribution of the EC_{50}s was unimodal, suggesting that no resistant subpopulation of *B. dothidea* was present among the isolates. The range-of-variation of 10.18-fold was the lowest compared with other fungicides reported (Song et al. 2018). For fludioxonil, the range-of-variation factor was 25, while the VF values of fluazinam and pyrisoxazole were both more than 50, which may reflect low intrinsic variability in sensitivity to pyraclostrobin among *B. dothidea* isolates.

QoI fungicides were first commercialized in 1996. FRAC categorizes QoI fungicides as being at high risk of resistance development (FRAC 2014) because one amino acid switching from glycine to alanine at position 143 (G143A) of the mitochondrial cytochrome b gene can confer high levels of resistance (Wise et al. 2008). Indeed, after only 2 to 4 years of commercialization, resistance was detected in field populations of many plant pathogen species (Gisi et al. 2002), including *Alternaria solani* (Pasche et al. 2004), *Botrytis cinerea* (Bardas et al. 2010; Kim and Xiao 2010), *C. beticola* (Birla et al. 2012), *Erysiphe graminis* (Chin et al. 2001; Sierotzki et al. 2000), *Venturia inaequalis* (Zheng and Olaya 2000), *Alternaria mali* (Lu et al. 2003), *Alternaria alternata* (Avenot et al. 2008), *Monilinia laxa* (Miessner and Stammler 2010), *Phytophthora cactorum* (Rebollar-Alviter et al. 2007), *Magnaporthe grisea* (Avila-Adame and Köller 2003), and *Sclerotinia sclerotiorum* (Di et al. 2016). Given that little information exists regarding ring rot control with pyraclostrobin, studies evaluating baseline sensitivities of *B. dothidea* to these chemistries should serve the apple industry well, and improve spray programs currently implemented by apple growers of China.

Fungicide resistance development mainly depends on the inheritance, displacement, as well as the pathogenic fitness of the resistant mutants (Sierotzki et al. 2000; Zhang et al. 2012b). Some mutations can be inherited even with no fungicide pressure (Bi and Ma 2016). In this study, after reproducing 15 generations on fungicide-free PDA medium or 4 generations on apples, the pyraclostrobin sensitivity and the pathogenicity of the resistant isolates remained similar. These results indicate that the pyraclostrobin resistance is stable with no or very low fitness cost. Our resistance stability and pathogenicity study supported the FRAC classification that pyraclostrobin is a fungicide with high resistance risk. Moreover, resistance to QoIs has developed more rapidly than expected for certain diseases, such as cucumber powdery mildew and downy mildew in Japan or wheat powdery mildew in Europe (Ishii et al. 2001; Sierotzki et al. 2000). However, other studies have indicated a possible decrease in fitness of the pyraclostrobin-resistant mutants (Brent and Hollomon 2007; Zhang et al. 2012b). Currently, there is no report regarding resistance development to other QoI fungicides in *B. dothidea*. Therefore, it is important to monitor any alteration of *B. dothidea* sensitivity to QoIs under field conditions.

The present study indicated no cross-resistance between pyraclostrobin and other popular fungicides carbendazim, tebuconazole,

flusilazole, and iprodione with different modes of action. *B. dothidea* isolates with reduced sensitivity to tebuconazole were also less sensitive to propiconazole, which has the same mode of action. Similarly, cross-resistance between tebuconazole and difenoconazole, two DMI-fungicides, was reported for *Didymella bryoniae* (Thomas et al. 2012). Cross-resistances between fungicides with the same mode of action are common, as a given resistance mechanism often can target multiple compounds of the same chemical group with the same mode of action (Zhang et al. 2012b). However, there are exceptions depending on the fungal species. For example, *Sclerotinia sclerotiorum* did not show cross resistance to two QoI fungicides trifloxystrobin and pyraclostrobin (Lu et al. 2015) and to different demethylation inhibitors (DMIs), while cross-resistance among these fungicides were reported for other fungi (Karaoglanidis et al. 2003; Thomas et al. 2012). These results demonstrate that it is necessary to characterize cross-resistance profile to aid selection of rotation partners in disease control to delay pesticide resistance (Fan et al. 2016b). The current study showed that pyraclostrobin can be used to manage *B. dothidea* that is resistant to carbendazim, tebuconazole, flusilazole, and iprodione.

Under field conditions, pyraclostrobin applications significantly reduced disease incidence compared with that of the untreated plots. The efficacy was similar to that of tebuconazole, carbendazim, and flusilazole. The QoI fungicide pyraclostrobin is well known for its high activity against a wide range of oomycete, ascomycete, basidiomycete, and deuteromycete pathogens (Di et al. 2016). Our field evaluation suggested that pyraclostrobin is an excellent fungicide and can be registered for the control of *B. dothidea* on apples in China. However, considering its high resistance risk classification, limiting the number of sprays, rotating with other fungicides of different modes of action, and timely monitoring of susceptibility should be implemented to avoid or delay resistance development. Furthermore, other integrated management tactics such as disease-resistant varieties, crop rotations, and biological control agents should also be implemented.

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