Evaluating the Sensitivities and Efficacies of Fungicides with Different Modes of Action Against *Phomopsis asparagi*

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Abstract

Asparagus stem blight caused by *Phomopsis asparagi* is a major hindrance to asparagus production worldwide. Currently, fungicides are used to manage the disease in commercial production, but resistance to common fungicides has emerged in the wild population. In the present study, 132 isolates of *P. asparagi* collected from different provinces in China were tested for sensitivities to pyraclostrobin, tebuconazole, and fluazinam. We also determined the efficacies of six fungicides against *P. asparagi*. The frequency distributions of EC₅₀ values of the isolates tested were unimodal, but the curves for pyraclostrobin and tebuconazole had long right-hand tails. The mean EC₅₀ values for pyraclostrobin, tebuconazole, and fluazinam were 0.0426 ± 0.0029, 0.6041 ± 0.0416, and 0.0314 ± 0.0013 µg/ml, respectively. In addition, the EC₅₀ values for pyraclostrobin were very similar with or without salicylhydroxamic acid (SHAM), 20 µg/ml, indicating that SHAM is not needed to determine the sensitivity of *P. asparagi* to pyraclostrobin when using the mycelial growth inhibition assay. In greenhouse assays, Merivon (42.4%

fluxapyroxad plus pyraclostrobin SC), Frown-cide (500 g/liter fluazinam SC), Cabrio (250 g/liter pyraclostrobin EC), and Nativo (75% trifloxystrobin plus tebuconazole WG) showed excellent preventive efficacy against *P. asparagi*. And these fungicides were more effective before inoculation than when they were applied after inoculation ($P < 0.05$). Therefore, these fungicides should be applied prior to infection to control stem blight. In field trials, Frown-cide, Merivon, Nativo, and Cabrio also performed good control effects, ranging from 75.2 to 86.0% in 2017 and 75.4 to 87.1% in 2018. We demonstrated that Frown-cide, Merivon, Nativo, and Cabrio had considerable potential to manage asparagus stem blight. In addition, rotations of these fungicides are essential for precluding or delaying the development of resistance and for controlling the disease.

Keywords: *Phomopsis asparagi*, control efficacy, diverse fungicides, baseline sensitivity, asparagus stem blight

Garden asparagus (*Asparagus officinalis* L.), known as the king of vegetables, is an economically important crop that has been cultivated for over 2,500 years. Asparagus has a high nutritional value with several health benefits including antioxidant, anti-inflammatory, and antihepatotoxic properties (Almehdar et al. 2012; Dehghan-Shahreza et al. 2016; Li et al. 2016; Rusjan and Mikulic-Petkovsek 2017). This vegetable has become an essential item in cuisine (Yang et al. 2016). Over 50% of the global cultivated asparagus area and volume occurs in China, which makes China currently the largest producer and exporter worldwide (Y. P. Zhang et al. 2012).

Stem blight disease caused by *Phomopsis asparagi* (Sacc.) Bubak has caused major setbacks in global asparagus production, particularly in humid areas (Davis 2001; Elena 2006). The disease has been reported in many Chinese provinces, including Fujian, Shandong, and Jiangxi (Meng et al. 2013). There are two high-yielding seasons for asparagus in the subtropical regions of China, spring (from mid-April to early June) and autumn (from mid-August to mid-October). Outbreaks of stem blight occur in both seasons. The disease infects the leaves and the stem. Typical symptoms are round to oval lesions that are pale in color. Pycnidia are

usually observed within older lesions. As the disease progresses, plants desiccate completely and the stems die (Elena 2006).

None of the known asparagus varieties on the market are resistant to stem blight. The breeding of resistant varieties is difficult and time-consuming because the species is dioecious and perennial (Sonoda et al. 1997). At present, fungicide application is still the primary method for managing stem blight disease. There are several fungicides registered against asparagus stem blight, such as difenoconazole, mancozeb, diniconazole, and thiophanate methyl, and so on (China Pesticide Information Network, <http://www.icama.org.cn/fwb/index.jhtml>). In the past, the fungicides thiophanate methyl, carbendazim, and mancozeb were used widely in China to manage the disease. Resistance within *P. asparagi* to the three fungicides has been reported in China (Chen and Ye 1999; Meng et al. 2013). As the disease continues to spread geographically, new and effective fungicides are essential for *P. asparagi* management.

The strobilurin fungicides (quinine outside inhibitors, QoIs) are derived from natural products of wood-rotting fungi, including *Oudemansiella mucida* and *Strobilurus tenacellus*. The strobilurin fungicides inhibit mitochondrial respiration by blocking electron transfer within the respiratory chain (Bartlett et al. 2002), which in turn causes severe disruption of important cellular biochemical processes and stops fungal growth. However, when normal respiration is inhibited in the presence of QoIs, an alternative mitochondrial respiratory pathway can be activated (Kaneko and Ishii 2009), which is mediated by alternative oxidase (AOX) in fungi (Xu et al. 2012). This pathway may result in inaccuracies in vitro evaluations of QoI sensitivity. Therefore, AOX should be inhibited during in vitro assays. AOX is resistant to cyanide, but sensitive to salicylhydroxamic acid (SHAM) and *n*-propyl gallate (*n*PG) (Schonbaum et al. 1971). Pyraclostrobin is a broad spectrum foliar fungicide in the strobilurin chemical class, which has protective, curative, and eradicated activities against major plant pathogens (Stammler et al. 2012; Yang and Bai 2012), such as *Ascochyta rabiei* (Wise et al. 2008) and *Fusarium* spp. (Broders et al. 2007; Munkvold 2009). We define protective activity as the fungicide is applied before the pathogen arrives or infects

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the host and the fungicide protects the plant from infection. Curative activity occurs when the fungicide is applied after infection and prevents growth of the pathogen early in infection of the host tissues. Eradicative activity occurs when the fungicide is applied after symptoms develop (Ivic 2010). Unfortunately, owing to a single site of action and a high resistance risk (Bartlett et al. 2002), pyraclostrobin resistance has been reported in many plant fungal pathogens (Liang et al. 2015b; G. R. Zhang et al. 2012).

The triazole fungicides are part of the demethylation inhibitor fungicide group (DMIs) (Hamamoto et al. 2000), and contain several commercially successful compounds, such as difenoconazole and tebuconazole, which target the heme iron of cytochrome P450 sterol 14 α -demethylase (CYP51) and interfere with ergosterol biosynthesis of cell membrane of pathogen (Yoshida and Aoyama 1987). Tebuconazole is a triazole fungicide, which was developed in the late 1990s by the Bayer Corporation. It has been extensively used on a wide range of crops in many countries because of its ability to control diverse fungal diseases (He et al. 2018; Liang et al. 2018; Paul et al. 2007; Yoshimura et al. 2004). Furthermore, it is also one of the most important and widely used fungicides for disease control in rice within China (Liu et al. 2013; Wang et al. 2007). However, the extensive application of tebuconazole has provoked reduced sensitivity in many plant pathogens (Shi et al. 2017).

Fluazinam belongs to the novel broad-spectrum phenylpyridine fungicide that acts at all developmental stages from spore germination to sporulation, and especially infection. It is considered a protectant and has a good persistent effect and rain fastness, but has little curative or systemic activity (fungicide can be absorbed by plants and transported to other parts of plants) (Liang et al. 2015a). Fluazinam is very effective against a large number of plant pathogens (Lemay et al. 2002; Smith et al. 2008; Wang et al. 2016), but it is not effective against rusts and powdery mildew (Guan et al. 2014; Liu and Zhang 2017). To date, fluazinam has been registered in China to manage *Phytophthora infestans* in potato (*Solanum tuberosum*) and *Plasmiodiophora brassicae* in Chinese cabbage (*Brassica pekinensis*) (Dowley and Sullivan 1995; Nielsen and Schepers 2014; Shi et al. 2016; Wang 2015).

Construction of baseline sensitivity is a significant part of the registration process for all fungicides in the European Union (Di et al. 2016), and baseline data are required by the Chinese registration authority (Fan et al. 2019). Pyraclostrobin, tebuconazole, and fluazinam have not yet been registered in China for the control of *P. asparagi* on asparagus (China Pesticide Information Network, <http://www.icama.org.cn/fwb/index.jhtml>). Thus, this is important to establish the baseline sensitivity of *P. asparagi* to these three fungicides and monitor fungicide resistance. Accordingly, we assessed the baseline sensitivities of *P. asparagi* to pyraclostrobin, tebuconazole, and fluazinam, determined the efficacies of six fungicides [Cabrio (250 g/liter pyraclostrobin EC), Frown-cide (500 g/liter fluazinam SC), Folicur (430 g/liter tebuconazole SC), Dithane M-45 (80%

mancozeb WP), Nativo (75% trifloxystrobin plus tebuconazole WG), and Merivon (42.4% fluxapyroxad plus pyraclostrobin SC)] against *P. asparagi* in a greenhouse and in the field.

Materials and Methods

Experimental material: *P. asparagi* isolates and asparagus cultivar. We collected 132 fungal isolates from two to three fields of naturally infected asparagus in each of seven Chinese provinces: Fujian, Shandong, Henan, Hunan, Shanxi, Jiangxi, and Zhejiang (Table 1). The sampled fields were separated by at least 100 km from one another. Twenty young stems from plants with typical symptoms were collected at random within each field. Symptomatic tissues were cut into 3–5 mm pieces, and surface-sterilized by immersion in 75% (vol/vol) ethanol for 30 s, followed by 1% NaClO for 1.5 min, then rinsed three times in distilled water, and incubated on potato dextrose agar (PDA) at 28°C. After 3–5 days, mycelia were transferred to fresh PDA plates at 28°C for 2–3 weeks to encourage pycnidial formation. Then single conidium isolations were made as described earlier (Meng 2013). All isolates were identified on the basis of morphology and partial sequence analysis of ITS, and preserved on filter paper and stored at –20°C.

The cultivar, 'Grande F1', which is susceptible to asparagus stem blight, was used to assess fungicide efficacy in greenhouse and field trials.

Fungicides. Technical grade products of each fungicide were used in the fungicide sensitivity assays. Pyraclostrobin (98% active ingredient [a.i.]; Jiangsu Huifeng Agrochemical Co., Ltd., Dafeng, China), tebuconazole (95% a.i.; Nantong Taihe Chemical Co., Ltd., Nantong, China), and fluazinam (97% a.i.; Ishihara Sangyo Kaisha, Osaka, Japan) were dissolved in dimethyl sulfoxide (DMSO) to produce stock solutions of 10,000 μ g/ml. Salicylhydroxamic acid (SHAM, 99% a.i.; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO to obtain a 9.6×10^4 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 fungicide sensitivity trials.

Commercially formulated fungicides containing Cabrio (250 g/liter pyraclostrobin EC, BASF China Ltd., Shanghai, China), Frown-cide (500 g/liter fluazinam SC, Japan Ishihara Sangyo Kaisha), Folicur (430 g/liter tebuconazole SC, Bayer Crop Science [China] Co., Ltd., Hangzhou, China), Dithane M-45 (80% mancozeb WP, Dow Agrosciences China Ltd., Nantong, China), Nativo (75% trifloxystrobin plus tebuconazole WG, Bayer Crop Science [China] Co., Ltd.), and Merivon (42.4% fluxapyroxad plus pyraclostrobin SC, BASF China Ltd., Shanghai, China) were dissolved in water.

Determination of EC₅₀ values and baseline sensitivities of *P. asparagi* to pyraclostrobin, tebuconazole, and fluazinam. We determined EC₅₀ values of fungicides using a mycelial growth inhibition assay based on the procedures described in Amir et al. (2013), with minor modifications. Potato dextrose agar (PDA) was serially amended with pyraclostrobin to give final concentrations

Table 1. Sensitivities of *Phomopsis asparagi* isolates to pyraclostrobin, tebuconazole, and fluazinam in China

Location	Number of isolates	EC ₅₀ for Pyraclostrobin (μ g/ml) ^x		EC ₅₀ for Tebuconazole (μ g/ml)		EC ₅₀ for Fluazinam (μ g/ml)	
		Range	Mean ^y	Range	Mean	Range	Mean
Fujian	23	0.0134–0.1532	0.0468 $\pm$ 0.0071 a	0.0821–2.5227	0.8664 $\pm$ 0.1440 a	0.0107–0.0620	0.0322 $\pm$ 0.0028 a
Henan	20	0.0125–0.1238	0.0506 $\pm$ 0.0099 a	0.0752–1.8736	0.6513 $\pm$ 0.1078 a	0.0081–0.0579	0.0316 $\pm$ 0.0032 a
Hunan	18	0.0108–0.0920	0.0341 $\pm$ 0.0049 a	0.0207–1.0621	0.4530 $\pm$ 0.0717 a	0.0048–0.0526	0.0329 $\pm$ 0.0042 a
Jiangxi	18	0.0107–0.1506	0.0449 $\pm$ 0.0095 a	0.0275–1.6178	0.5452 $\pm$ 0.0958 a	0.0067–0.0549	0.0299 $\pm$ 0.0035 a
Shanxi	19	0.0111–0.1511	0.0474 $\pm$ 0.0083 a	0.1098–2.1180	0.6273 $\pm$ 0.1126 a	0.0115–0.0533	0.0320 $\pm$ 0.0029 a
Shandong	17	0.0120–0.1006	0.0357 $\pm$ 0.0050 a	0.0542–1.3406	0.4998 $\pm$ 0.0839 a	0.0068–0.0628	0.0295 $\pm$ 0.0038 a
Zhejiang	17	0.0098–0.1305	0.0359 $\pm$ 0.0070 a	0.0663–1.2314	0.4941 $\pm$ 0.0819 a	0.0058–0.0615	0.0312 $\pm$ 0.0041 a
Total	132	0.0098–0.1532	0.0426 $\pm$ 0.0029	0.0207–2.5227	0.6041 $\pm$ 0.0416	0.0048–0.0628	0.0314 $\pm$ 0.0013
VF ^z		15.63		121.87		13.08	

^x EC₅₀, the effective concentration of the fungicide causing 50% inhibition of mycelial growth on PDA media.

^y Mean $\pm$ SEM (standard error of the mean), different lowercase letters identify significantly different means in columns (Tukey's multiple comparisons test; $P = 0.05$).

^z VF = (Maximum EC₅₀) / (Minimum EC₅₀)

of 0.00625, 0.0125, 0.025, 0.05, 0.1, and 0.5 µg a.i./ml. Similarly, PDA was amended with tebuconazole at final concentrations of 0.02, 0.04, 0.2, 1, 5, and 20 µg a.i./ml, or fluazinam at final concentrations of 0.00625, 0.0125, 0.025, 0.05, and 0.1 µg a.i./ml. PDA plates amended with 0.1% (vol/vol) DMSO served as control. A 5-mm mycelial plug of each *P. asparagi* isolate was cut from the periphery of a 5-day-old colony growing on PDA and placed upside down onto the center of fungicide-amended PDA plates. After 7–9 days of incubation at 28°C in the dark, we measured the colony diameter of each isolate twice at right angle coordinate positions. Each fungicide concentration had five replicates and the experiments were conducted twice.

Effect of SHAM on mycelial growth of *P. asparagi*. A stock solution of SHAM was diluted and added to PDA media to obtain final concentrations of 10, 20, 40, 80, 160, and 320 µg/ml, respectively. PDA amended with 0.1% (vol/vol) DMSO served as a control. Ten isolates that differed in EC₅₀ values to pyraclostrobin were selected and tested for the toxic effects of SHAM on mycelial growth. Isolates were prepared for plating using the methods described above. The experiments were performed three times.

The EC₅₀ values of pyraclostrobin in the presence of SHAM (at a concentration of 20 µg/ml) were also assessed on pyraclostrobin-amended PDA. Control plates were prepared with DMSO only.

Preventive and curative efficacies of six fungicides against *P. asparagi* in a greenhouse. Ten isolates from seven provinces were used in this study (Table 2). The 10 isolates were incubated at 28°C on PDA plates for 2–3 weeks to encourage pycnidial formation. Ten to 15 ml of distilled water were added to the plates and the surface of the colonies was gently washed with a paintbrush. The conidial suspension of a mixture of 10 isolates was filtered through two layers of cheesecloth and adjusted to 1 × 10⁵ conidia/ml with 0.1% aqueous solution Tween 80 surfactant. Asparagus seeds were soaked in hot water (55–58°C) for 20 min, rinsed in cold water, and then immersed in 50% carbendazim WP at 500 µg a.i./ml for 48 h. The seeds were subsequently germinated for 72 h at 28°C and 100% humidity, then one seed was sown in a polypropylene pot containing sphagnum peat soil (Pindstrup, Ryomgaard, Denmark). The plants were incubated at 24–28°C in a greenhouse (12 h light and RH > 90%). To determine the efficacy of a preventative application of a fungicide, seedlings were first sprayed with dilutions of Cabrio at rates of 84.38, 112.5, and 168.75 g a.i./ha, Frown-cide at 187.50, 225.00, and 262.50 g a.i./ha, Folicur at 64.50, 96.75, and 129.00 g a.i./ha, Dithane M-45 at 1350.00, 1800.00, and 2700.00 g a.i./ha, Nativo at 33.75 + 67.50, 42.19 + 84.38, and 56.25 + 112.50 g a.i./ha, and Merivon at 57.24 + 57.24, 71.55 + 71.55, and 95.40 + 95.40 g a.i./ha, respectively, using a knapsack sprayer (Matabi super green16). Then the plants were inoculated with a conidial suspension (1 × 10⁵ conidia/ml) described above 24 h after fungicide application. To determine the efficacy of a curative application on a fungicide,

a conidial suspension (1 × 10⁵ conidia/ml) was first sprayed on the asparagus seedlings, then fungicide treatments were applied to the seedlings 24 h after inoculation. Control plants were sprayed with a 0.1% Tween 80 solution. The treated plants and control plants were maintained under greenhouse conditions as described above. Each dose of each fungicide contained 20 plants, each of which had three replicates. All experiments were conducted in triplicate.

Disease severity was evaluated on a 0–9 scale according to the evaluation system of Yang et al. (2012) with slight modifications, where: 0, no evidence of infection; 1, ≤5% of the stem surface diseased; 3, 5 to 15% of the stem surface diseased; 5, 16 to 25% of the stem surface diseased; 7, 26 to 50% of the stem surface diseased; 9, ≥50% of the stem surface diseased. The disease index and control efficacy for each fungicide were calculated according to the following formulas (Dai et al. 2018).

Disease index

$$= \frac{\sum(\text{the number of diseased plants in this scale} \times \text{the disease scale})}{\text{the total plants investigated} \times 9} \times 100$$

Control efficacy(%)

$$= \frac{\text{disease index of control} - \text{disease index of treated}}{\text{disease index of control}} \times 100$$

Control efficacies of six fungicides against asparagus stem blight in field trials. A field with a history of stem blight, located in Zhuangbian Town, Hanjiang District, Putian, Fujian Province, China, was used for this experiment. Trials were performed on 3-year-old plants of the asparagus hybrid cv. ‘Grande F1’ in 2017 and 2018. Plants were spaced at 25-cm intervals and the row spacing was 1.5 m. Per plot consisted of 30 m² (3 m wide × 10 m long). The trial contained 19 treatments in a randomized block design with three replications. The same fungicides and rates used in a greenhouse were applied until runoff to the plants in the field using knapsack sprayer (Matabi super green16). Control plants were sprayed with water. In 2017, fungicides were first applied on 5 September 2017, with subsequent applications at 7-day intervals for 2 weeks; in 2018, fungicides were first applied on 28 April 2018, with subsequent applications at 7-day intervals for 2 weeks. Disease severity was measured 14 days after the last application. The disease index and control efficacy were calculated as described above.

Statistical analyses. The fungicide concentrations and corresponding inhibitions were used to calculate EC₅₀ values with the Bio-assay Analysis for Quantitative Data in Data Processing System software (ver. 7.05; Hangzhou RuiFeng Information Technology Co. Ltd., Hangzhou, China). Baseline sensitivity was calculated from

Table 2. EC₅₀ values for 10 *Phomopsis asparagi* isolates on pyraclostrobin-amended PDA with and without SHAM

Isolate	Location	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	
sd34	Shandong	0.0179	0.0097–0.0331	0.0221	0.0130–0.0378	...
zb15	Fujian	0.0297	0.0237–0.0371	0.0534	0.0387–0.0742	*
hn30	Hunan	0.0145	0.0056–0.0377	0.0148	0.0061–0.0362	...
qd53	Jiangxi	0.0392	0.0214–0.0717	0.0421	0.0219–0.0806	...
zj28	Zhejiang	0.0370	0.0304–0.0450	0.0357	0.0281–0.0476	...
hn34	Henan	0.0130	0.0070–0.0242	0.0151	0.0110–0.0208	...
sx37	Sanxi	0.0112	0.0054–0.0233	0.0229	0.0178–0.0294	...
sd20	Shandong	0.0194	0.0081–0.0463	0.0233	0.0165–0.0329	...
zb07	Fujian	0.0092	0.0058–0.0148	0.0108	0.0079–0.0148	...
jx43	Jiangxi	0.0133	0.0082–0.0215	0.0152	0.0081–0.0288	...
Average		0.0204		0.0255		P = 0.057 ^z

^w Final concentration of SHAM in PDA media was 20 µg/ml.

^x The 95% confidence intervals (CIs) were calculated using Data Processing System software (ver. 7.05; Hangzhou RuiFeng Information Technology Co. Ltd.).

^y * denotes that there was not significant difference in EC₅₀ values between pyraclostrobin with and without SHAM.

^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.

the frequency distributions of EC_{50} values for the 132 isolates following the procedures of Russell (2004). Pairwise significant differences in mean EC_{50} values between different *P. asparagi* populations were identified using Tukey's multiple comparisons test in one way analysis of variance (ANOVA). Student's paired *t* test was employed for significant difference in the mean EC_{50} values between pyraclostrobin with and without SHAM, and between preventive and curative efficacies. Preventive and curative efficacies in a greenhouse, and control efficacies in a field were subjected to ANOVA using Duncan's new multiple-range test (DMRT) ($P \leq 0.05$). All data were arcsine square-root transformed prior to statistical analyses (McDonald 2009).

Results

Baseline sensitivity of *P. asparagi* to pyraclostrobin, tebuconazole, and fluazinam. The EC_{50} values of 132 *P. asparagi* isolates collected from different provinces of China for pyraclostrobin, tebuconazole, and fluazinam ranged from 0.0098–0.1532 $\mu\text{g/ml}$, 0.0207–2.5227 $\mu\text{g/ml}$, and 0.0048–0.0628 $\mu\text{g/ml}$, respectively, and the respective mean EC_{50} values were 0.0426 ± 0.0029 $\mu\text{g/ml}$, 0.6041 ± 0.0416 $\mu\text{g/ml}$, and 0.0314 ± 0.0013 $\mu\text{g/ml}$ (Table 1). The EC_{50} ranges for pyraclostrobin and fluazinam were narrow, while a broader range of sensitivity values was detected for tebuconazole with the variation factor [*VF*, (maximum EC_{50}) / (minimum EC_{50})] of 121.87 (Table 1). There was no significant difference ($P = 0.6207, 0.9938, 0.085$, respectively) in sensitivities (mean EC_{50} values) among the seven populations for pyraclostrobin, fluazinam, and tebuconazole (Table 1).

Fig. 1. Frequency distributions of sensitivity values (EC_{50}) for 132 isolates of *Phomopsis asparagi* to pyraclostrobin (A), tebuconazole (B), and fluazinam (C).

The frequency distributions of sensitivities to pyraclostrobin and tebuconazole were similar in shape, unimodal with a long right-hand tail (Fig. 1A and B). The frequency distribution of sensitivities to fluazinam fit a normal curve ($P = 0.8517$, Kolmogorov-Smirnov goodness-of-fit test) (Fig. 1C).

Toxic effects of SHAM on the mycelial growth of *P. asparagi*.

Mycelia of the four isolates (zb30, hn34, sx37, and sd20) growing on PDA plates had a quantitative dose response to SHAM, with EC_{50} values of 306.75, 117.54, 118.16, and 132.05 $\mu\text{g/ml}$, respectively (Fig. 2). All isolates grew well at a SHAM concentration of 10 $\mu\text{g/ml}$, but significant growth inhibition occurred at concentrations ≥ 40 $\mu\text{g/ml}$. The results demonstrated that SHAM at concentrations ≤ 20 $\mu\text{g/ml}$ had inapparent toxic effects on the mycelial growth of *P. asparagi*.

Effects of SHAM on mycelial growth of *P. asparagi* on PDA. In pyraclostrobin-amended PDA, the EC_{50} values of the 10 isolates tested were similar with and without SHAM (20 $\mu\text{g/ml}$) (Table 2). The average EC_{50} value across the 10 isolates was 0.0255 $\mu\text{g/ml}$ for pyraclostrobin without SHAM, and 0.0204 $\mu\text{g/ml}$ with SHAM. Mean EC_{50} values between pyraclostrobin with and without SHAM were not significantly different ($P = 0.057$) according to a paired *t* test.

Preventive and curative efficacies of six fungicides against *P. asparagi* in a greenhouse. Merivon, Frown-cide, Cabrio, and Nativio showed excellent preventive efficacy against *P. asparagi* (Table 3). The highest concentration of Merivon (95.40 + 95.40 g a.i./ha), Frown-cide (262.50 g a.i./ha), Cabrio (168.75 g a.i./ha), and Nativio (56.25 + 112.50 g a.i./ha) reduced disease severity by 87.9, 87.3, 86.7, and 85.5%, respectively, 24 h prior to inoculation, and there was significant difference ($P < 0.05$) with the efficacy of Dithane M-45 at a rate of 2700.00 g a.i./ha, which was 74.7%. Compared with the preventive activities, the curative activities of all fungicides tested were lower at the same concentrations according to Student's paired *t* test ($P < 0.05$).

Control efficacies of six fungicides against asparagus stem blight in field trials. Disease index in the untreated controls was 19.7% in 2017 and 31.0% in 2018 (Table 4). The disease severity values in all treatments were significantly lower than those in the untreated controls ($P < 0.05$). Among six fungicides, Frown-cide and Merivon exhibited better control efficacy at 262.50 and 95.40 + 95.40 g a.i./ha compared with other fungicides at the highest dose during 2 years, and reduced disease severity by 86.0 and 85.3% in 2017, and 87.1 and 86.1% in 2018, respectively. Cabrio at a rate of 168.75 g a.i./ha was not significantly different ($P > 0.05$) from the efficacy of Nativio at 56.25 + 112.50 g a.i./ha during 2 years. The control efficacy of Dithane M-45 at 2700.00 g a.i./ha was significantly different ($P < 0.05$) from that of five other fungicides tested at the highest dose. In general, the efficacies of fungicides tested were

Fig. 2. Mycelial growth inhibitions of *Phomopsis asparagi* by increasing concentrations of salicylhydroxamic acid (SHAM) on PDA media. The error bar indicates standard deviation (SD) of four replicates.

relatively stable during the two consecutive years, ranging from 67.8 to 86.0% in 2017 and 68.7 to 87.1% in 2018.

Discussion

Establishment of baseline sensitivities and corresponding discriminatory doses are crucial, especially in the light of fungicide resistance development. In the current study, 132 isolates of *P. asparagi* collected from seven provinces of China were tested for sensitivities to three fungicides that have yet to be registered for use on asparagus in China. Technically, baseline sensitivity should be established by testing a target pathogen without an exposure history (Russell 2004). The sensitivity data obtained in our study cannot be considered as definitive baselines but rather relative baselines because it is not clear whether the isolates tested had been previously exposed to other fungicides with the same mechanism.

Nevertheless, these relative baseline sensitivities will be valuable for monitoring resistance levels in *P. asparagi* populations in China in the future (Russell 2002). Most of the isolates tested were sensitive to the three fungicides, and the frequency distribution of each fungicide was unimodal. Moreover, the sensitivity frequency distributions of pyraclostrobin and tebuconazole had long right-hand tails on the sensitivity distribution curve. A long right-hand tail on a sensitivity distribution curve may indicate a strong potential for future resistance. In fact, resistance development for strobilurins (QoI fungicides) and triazoles (DMI fungicides) has been reported in many pathogens (Ahmed et al. 2014; Banno et al. 2009; Milgate et al. 2016). Although Fungicide Resistance Action Committee (FRAC) has listed fluazinam as a low-risk fungicide, resistance to fluazinam has been reported in *Ustilago maydis* (FRAC 2014; Vitoratos 2014). Therefore, monitoring *P. asparagi* populations

Table 3. Preventive and curative activities of six fungicides against asparagus stem blight in a greenhouse

Fungicides	Active ingredients	Chemical group	Product rate (a.i. g ha ⁻¹)	Preventive efficacy (%) ^x	Curative efficacy (%) ^y
Cabrio	Pyraclostrobin	Strobilurin	84.38	79.2 defg ^z	72.2 fgh
			112.50	81.7 cde	75.2 cdef
			168.75	86.7 ab	77.2 bcde
Frown cide	Fluazinam	Pyridines	187.50	81.7 cde	73.2 efgh
			225.00	83.6 abcd	76.5 cde
			262.50	87.3 a	80.8 ab
Folicur	Tebuconazole	Triazole	64.50	75.4 fgh	70.3 gh
			96.75	77.7 efg	74.0 defg
			129.00	79.8 def	78.5 abc
Dithane M-45	Mancozeb	Organosulfur	1350.00	69.4 i	62.2 j
			1800.00	72.2 hi	65.9 ij
			2700.00	74.7 gh	69.3 hi
Nativo	Trifloxystrobin+Tebuconazole	Strobilurin+Triazole	33.75+67.50	78.5 efg	70.1 gh
			42.19+84.38	82.2 bcde	73.3 efgh
			56.25+112.50	85.5 abc	77.1 bcde
Merivon	Pyraclostrobin+Fluxapyroxad	Strobilurin+SDHI	57.24+57.24	81.5 cde	73.7 efg
			71.55+71.55	84.8 abc	78.1 bcd
			95.40+95.40	87.9 a	82.2 a

^x Fungicides for preventive efficacy were applied 24 h before inoculation.

^y Fungicides for curative efficacy were applied 24 h after inoculation.

^z Mean ± SEM (standard error of the mean); values in the same column followed by different lowercase letters are significantly different according to Duncan's new multiple-range test ($P \leq 0.05$).

Table 4. Control efficacies of six fungicides against asparagus stem blight in 2017 and 2018 field trials

Treatment	Active ingredients	Chemical group	Product rate (a.i. g ha ⁻¹)	Disease index		Control efficacy (%) ^y	
				2017	2018	2017	2018
Non-treated control				19.7 a	31.0 a		
Cabrio	Pyraclostrobin	Strobilurin	84.38	4.9 cd	6.9 def	75.2 ij ^z	77.8 efg
			112.50	3.9 efg	6.1 fgh	80.0 ef	80.3 de
			168.75	3.3 hij	5.3 ghi	83.5 c	82.9 bc
Frown cide	Fluazinam	Pyridines	187.50	4.2 ef	6.2 efg	78.8 fg	80.0 de
			225.00	3.5 ghi	4.7 hi	82.1 cd	84.8 ab
			262.50	2.8 j	4.0 i	86.0 a	87.1 a
Folicur	Tebuconazole	Triazole	64.50	5.0 cd	7.6 cde	74.6 jk	75.3 gh
			96.75	4.5 de	7.1 def	77.1 gh	77.0 fgh
			129.00	4.1 efg	6.0 fgh	79.3 ef	80.6 cd
Dithane M-45	Mancozeb	Organosulfur	1,350.00	6.3 b	9.7 b	67.8 m	68.7 j
			1,800.00	5.9 b	8.8 bc	70.0 l	71.7 i
			2,700.00	5.2 c	8.0 cd	73.3 k	74.5 h
Nativo	Trifloxystrobin+Tebuconazole	Strobilurin+Triazole	33.75+67.50	4.5 de	7.6 cde	76.9 hi	75.4 gh
			42.19+84.38	3.8 fgh	6.4 efg	80.6 de	79.4 def
			56.25+112.50	3.2 ij	5.2 ghi	83.8 bc	83.0 bc
Merivon	Pyraclostrobin+Fluxapyroxad	Strobilurin+SDHI	57.24+57.24	4.1 efg	6.4 efg	79.1 ef	79.4 def
			71.55+71.55	3.2 ij	5.2 ghi	83.9 bc	83.3 b
			95.40+95.40	2.9 j	4.3 i	85.3 ab	86.1 a

^y Control efficacies of six fungicides against asparagus stem blight was evaluated on the 3-year-old plants of the asparagus hybrid cv. Grande F1.

^z Mean ± SEM (standard error of the mean); values in the same column indicate significant difference by Duncan's new multiple-range test ($P \leq 0.05$).

for sensitivity shifts to pyraclostrobin, tebuconazole, and fluazinam treatments in the future is essential to ensure these fungicides will continue to manage asparagus stem blight effectively.

The AOX pathway may result in inaccuracies in vitro sensitivity evaluations of some fungi to the QoIs. Consequently, SHAM, an inhibitor of the pathway, is often added to fungicide sensitivity assays to inhibit AOX (Duan et al. 2012; Xu et al. 2012). However, there are numerous reports of SHAM affecting the sensitivity of fungi to QoI fungicides in different ways. In some reports, SHAM at 100 µg/ml decreased EC₅₀ values of *S. sclerotiorum* to QoI fungicides (Duan et al. 2012). In others, the EC₅₀ values of *Fusicladium effusum* and *Microdochium majus* to azoxystrobin were higher in the presence of SHAM than that of azoxystrobin alone (Seyran et al. 2010; Walker et al. 2009). In our study, we detected similar EC₅₀ values for pyraclostrobin with and without SHAM at 20 µg/ml. Therefore, we suggest that SHAM is not needed to determine the sensitivity of *P. asparagi* to pyraclostrobin on mycelial growth inhibition assay.

Differences in efficacy among the six fungicides occurred in both field and greenhouse trials. In the greenhouse studies, Merivon, Frown-cide, Cabrio, and Nativio provided the higher preventive and curative efficacies against *P. asparagi* than all other fungicides tested. The lowest preventive and curative efficacies were observed with Dithane M-45. Overall, the preventive efficacies of fungicides tested were higher than their respective curative efficacies under the same conditions in the greenhouse ($P < 0.05$), indicating that these fungicides should be applied as preventive protectants before infections occur, rather than as curative fungicide after infection to control *P. asparagi* (Smith et al. 2008). Indeed, our results are similar to other studies that reported DMI fungicides, QoI fungicides, and fluazinam should be applied as preventive measures against *S. sclerotiorum* (Di et al. 2016; Li et al. 2015; Liang et al. 2015a). In the field trials, the rainfall (170.1 mm) from 28 April to 25 May 2018 far exceeded that (49.8 mm) from 5 September to 2 October 2017 in Zhuangbian Town. Accordingly, the disease incidence of the control group in 2018 was higher than that in 2017. Humid and rainy weather conditions are favorable for asparagus stem blight development (Davis 2001). Consistent with the results in the greenhouse, the lowest efficacy occurred in Dithane M-45, although this fungicide still reduced disease compared to the nontreated controls in the field. Among six fungicides, Frown-cide, Merivon, Nativio, and Cabrio reduced asparagus stem blight the most; therefore, these fungicides could be alternative fungicides to manage *P. asparagi* that is resistant to thiophanate methyl, carbendazol, and mancozeb. At present, although the resistance of *P. asparagi* to pyraclostrobin, fluazinam, and tebuconazole remains unreported, DMIs and QoIs are considered to be a medium-to-high risk for developing resistance. Accordingly, limiting the number of sprays, rotating with fungicides of different modes of action, and timely monitoring of susceptibility are essential for avoiding or delaying the development of resistance and maintaining efficacy.

Asparagus stem blight continues to spread as areas of cultivation expand to other countries (Davis 2001; Elena 2006; Zaw et al. 2017). It is now a devastating disease globally (Zhang et al. 2017). Although responsible application of highly effective fungicides has an important role in controlling stem blight, management of the disease requires an integrated approach based on seed treatment, water control, sound nutrition, host-plant resistance, and biological control. New cultural strategies will enable producers to continue supplying the market with high-quality asparagus in the future.

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