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Baseline sensitivity and efficacy of trifloxystrobin against *Sclerotinia sclerotiorum*

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ABSTRACT

The ascomycete fungus *Sclerotinia sclerotiorum* is a devastating plant pathogen notorious for its extremely broad host range. Trifloxystrobin has not been registered for control of *S. sclerotiorum* in China. In this study, baseline sensitivity of trifloxystrobin was established based on frequency distribution of 166 isolates' EC_{50} values and efficacy of trifloxystrobin was determined on potted oilseed rape plants. Trifloxystrobin EC_{50} values of the 166 isolates ranged from 0.01 to 0.80 μg/mL, with a mean value of 0.06 μg/mL. The frequency distribution of trifloxystrobin EC_{50} values was unimodal in shape, but with a long right-hand tail. After logarithmic transformation, the frequency distribution fitted closer to a normal distribution than did the original EC_{50} values. The preventive efficacies of trifloxystrobin at 5, 15, and 45 μg/mL were 71.4%, 96.5%, and 100.0%, respectively, while the curative efficacies were 40.6%, 48.7%, and 73.4%, respectively. Both preventive and curative efficacies of trifloxystrobin at 45 μg/mL were significantly higher ($P \leq 0.046$) than those of the reference fungicide carbendazim. Assays with six arbitrarily selected isolates demonstrated that salicylhydroxamic acid (SHAM) at 20 μg/mL reduced trifloxystrobin EC_{50} values by 84.0% on average and greatly potentiated efficacy of trifloxystrobin. The average co-toxicity factor of SHAM and trifloxystrobin for preventive efficacy was 64.3, indicating considerable synergisms *in planta*. Hence, SHAM should not be included in *in vitro* assay of *S. sclerotiorum* sensitivity to trifloxystrobin.

1. Introduction

The ascomycete fungus *Sclerotinia sclerotiorum* (Lib.) de Bary is a highly destructive plant pathogen that can infect more than 400 species of plants worldwide (Boland and Hall, 1994; Bolton et al., 2006). This fungus causes diseases commonly known as white mold, Sclerotinia stem rot, Sclerotinia head rot, stalk rot or wilt on a variety of broadleaf crops. The estimated yield losses of oilseed rape due to Sclerotinia stem rot range from 10% to 80% annually in China (Wang et al., 2014b). In the United States, annual economic loss in five crops attributed to Sclerotinia damage has been as high as $482 million, of which $300 million on soybean, $100 million on sunflower, $46 million on dry edible bean, $24 million on canola, and $12 million on pulse crop (http://www.ars.usda.gov/Research/docs.htm?docid=20122). Diseases caused by *S. sclerotiorum* have traditionally been difficult to control because resistant cultivars have not yet been adequately developed in major crops. In China, application of fungicides is still the principal method for control of *S. sclerotiorum* on oil crops such as oilseed rape, soybean, and sunflower (Wang et al., 2014b). Because of extensive and repeated applications of a relatively limited number of fungicides, fungicide resistance has arisen in this pathogen. By the end of the 1990s, high levels and prevalence of carbendazim resistance had been reported in oilseed rape fields in eastern China, leading to suspension of carbendazim in Sclerotinia stem rot management programs in Jiangsu Province (Pan, 1998; Shi et al., 2000; Wang et al., 2014b; Xu et al., 2015; Zhu et al., 2016). In 2009, a low level of dimethachlon resistance in *S. sclerotiorum* was reported in eastern China (Ma et al., 2009). More recently, medium to high levels of resistance to dimethachlon have been reported in Shaanxi Province of northwestern China, Heilongjiang Province of northeastern China, and Hunan Province of central China (Zhou et al., 2014a,b). With the development of resistance to commonly used fungicides, it is necessary to find appropriate alternative fungicides and to develop a more judicious fungicide-application program for control of *S. sclerotiorum*.

The quinone outside inhibitors (QoI) fungicides are highly

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effective in inhibiting sporulation, spore germination, and mycelial growth of many fungal plant pathogens. Owing to their broad spectrum of activity, QoI fungicides are used on a wide range of crops, such as cereals, pome fruits, tomatoes, potatoes, and cucurbits (Bartlett et al., 2002; Wise et al., 2008). As of early 2016, no QoI fungicide has been formally registered for control of *S. sclerotiorum* in China (China Pesticide Information Network, http://www.chinapesticide.gov.cn). Nevertheless, fungicidal activity and efficacy of the QoI fungicides azoxystrobin (Duan et al., 2012), benzothiostrobin (Xu et al., 2014) and pyraclostrobin (Liang et al., 2015a) against *S. sclerotiorum* have been reported. Trifloxystrobin, a member of the QoI group, has translaminar and redistribution properties in plants and can provide excellent protective and curative efficacy against a wide range of plant pathogens. Furthermore, trifloxystrobin has a favorable toxicological profile and is unlikely to cause any undue hazard to non-target organisms (Margot et al., 1998).

Construction of baseline sensitivity is essential for subsequent resistance monitoring programs and has become a significant part of the registration process for all fungicides in the European Union (Russell, 2004). When conducting *in vitro* assay of QoI fungicide sensitivity, salicylhydroxamic acid (SHAM), a characteristic inhibitor of the alternative oxidase (AOX), is routinely included in artificial culture media to suppress AOX (Duan et al., 2012; Malandrakis et al., 2006; Markoglou et al., 2006). However, our previous study indicated that in assay of *S. sclerotiorum* sensitivity to the QoI fungicide pyraclostrobin, SHAM should not be included in artificial media (Liang et al., 2015a). Whether SHAM should be added in assay of sensitivity to trifloxystrobin remains unknown. The objectives of this study were to (a) establish the baseline sensitivity of *S. sclerotiorum* to trifloxystrobin, (b) assess preventive and curative efficacies of trifloxystrobin, and (c) evaluate whether SHAM should be included in assay of trifloxystrobin sensitivity in *S. sclerotiorum*.

2. Materials and methods

2.1. Isolates of S. sclerotiorum

A total of 166 isolates were collected in 2013 from the following hosts and regions: 72 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 32 isolates from soybean fields in Heilongjiang Province of northeastern China. In each province, mature sclerotia were sampled from five or six fields at a distance of at least 20 km from each other. In each field, several plants with typical symptoms of Sclerotinia stem rot were arbitrarily selected. Sclerotia were air-dried and stored at 4 °C.

2.2. Fungicides

All fungicides used in the study were technical grade products. Trifloxystrobin (97% active ingredient [a.i.], Hubei Jianyuan Chemical Co. Ltd.), pyraclostrobin (95% a.i., Hubei Kang Bao Tai Fine-Chemical Co. Ltd.), and epoxiconazole (95% a.i., Hubei Jianyuan Chemical Co. Ltd.) were dissolved in acetone to produce their respective 1000 μg/mL stock solutions. Carbendazim (98.1% a.i., Haili Guixi Chemical Co. Ltd.) was dissolved in 0.1 mol/L hydrochloric acid (HCl) to produce a 1000 μg/ml stock solution. These stock solutions were stored at 4 °C, and in less than 2 weeks were serially diluted for bioassay experiments.

2.3. Determination of EC_{50} values and construction of baseline sensitivity

Determination of the effective concentration for 50% inhibition of mycelial growth (EC_{50}) was conducted according to Liang et al. (2015a), with minor modifications. Briefly, potato dextrose agar (PDA) media were amended with trifloxystrobin at final concentrations of 0.005, 0.01, 0.03, 0.1, 0.3, and 1.0 μg/mL. 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. PDA amended with solvent acetone at 0.2% by volume was used as the non-treated control. PDA plates were incubated at 23 °C in the dark for 48 h. The diameter of each colony was measured twice at right angles. The experiment was performed in triplicate. Trifloxystrobin concentrations and corresponding percentage inhibitions were used to calculate EC_{50} values by the procedure of Bioassay Analysis for Quantitative Data in the software Data Processing System (DPS, ver. 7.05; Hangzhou RuiFeng Information Technology Co. Ltd.). Baseline sensitivity of trifloxystrobin was constructed based on frequency distribution of EC_{50} values of the 166 isolates. For comparison purpose, baseline sensitivity was also established with logarithmically transformed EC_{50} values.

2.4. Sensitivity correlation of trifloxystrobin with pyraclostrobin and epoxiconazole

Sensitivity to pyraclostrobin and epoxiconazole in 16 arbitrarily selected isolates from different regions was assayed with a similar procedure as described above. Correlation coefficients (r) between sensitivity to trifloxystrobin and sensitivity to pyraclostrobin and epoxiconazole were calculated by the correlation procedure in GraphPad Prism (version 5.01; GraphPad Software, Inc.).

2.5. Preventive and curative efficacies of trifloxystrobin on potted oilseed rape plants

About 4-week-old oilseed rape plants growing in 20-cm-diameter plastic pots containing a composite mixture of peat and soil (1:1, wt/wt) at 26 ± 1 °C in growth chambers were used for efficacy assays. Stock solutions of trifloxystrobin and the reference fungicide carbendazim were diluted with 0.1% Triton-100 in water. Dilutions of trifloxystrobin at concentrations of 5, 15, and 45 μg/mL, and carbendazim at 45 μg/mL were sprayed with a hand-held sprayer (Xinmeir Co. Ltd., Zhejiang, China) on oilseed rape plants until run-off. Oilseed rape plants sprayed with 0.1% Triton-100 in water were used as the non-treated control. For determination of preventive efficacy, the treated plants were air-dried for 1 h, then inoculated on the adaxial surfaces of leaves (two leaves per plant) with inverted mycelial plugs. For determination of curative efficacy, oilseed rape plants were inoculated with mycelial plugs and incubated at 26 °C for 18 h, then sprayed with fungicide dilutions as described above. The inoculated plants were incubated at 26 °C in a growth chamber (photoperiod of 16 h) with relative humidity maintained over 85%. Lesion diameters were measured twice at right angles after 48 h of incubation for preventive efficacy and 48 h after fungicide application for curative activity. The experiment was performed in triplicate with treatments arranged in a completely randomized design.

2.6. Effect of SHAM on EC_{50} values of trifloxystrobin

Technical grade SHAM (99% a.i., Sigma-Aldrich) was dissolved in methanol to produce a stock solution of 10,000 μg/mL. For determination of trifloxystrobin EC_{50} values in the presence of SHAM,