Activity of the Novel Succinate Dehydrogenase Inhibitor Fungicide Pydiflumetofen Against SDHI-Sensitive and SDHI-Resistant Isolates of *Botrytis cinerea* and Efficacy Against Gray Mold

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Abstract

Succinate dehydrogenase inhibitor (SDHI) fungicides are currently the most frequently used fungicides for controlling gray mold. However, isolates of *Botrytis cinerea* resistant to SDHI fungicides have emerged in the field. Pydiflumetofen is a new SDHI fungicide that can control a variety of fungal diseases, but its efficacy against gray mold and whether the activity of pydiflumetofen is affected by the current SDHI-resistant isolates is currently unknown. The sensitivity of 291 single-spore *B. cinerea* isolates collected from 2017 to 2019 to pydiflumetofen was determined by spore germination inhibition assays. The mean EC₅₀ value (fungicide concentration resulting in a 50% inhibition compared with that of the control) of pydiflumetofen was 0.06 ± 0.01, 0.07 ± 0.02, and 0.05 ± 0.02 mg/liter in 2017, 2018, and 2019, respectively. There was no significant difference in the sensitivity of *B. cinerea* to pydiflumetofen among the 3 years. Furthermore, pydiflumetofen at 300 mg/liter effectively controlled gray mold on cucumber leaves (80.9%), and its efficacy was

superior to that of boscalid at 400 mg/liter (42.7%). The isolates carrying P225F, N230I, H272Y, and H272R mutations in the *SdhB* subunit were associated with the less sensitivity of *B. cinerea* to SDHI fungicides. After establishing the baseline sensitivity of *B. cinerea* to pydiflumetofen (EC₅₀ of 0.03 ± 0.003 mg/liter), we found that the P225F and H272Y mutant isolates showed low to moderate levels of resistance to pydiflumetofen, and the H272R and N230I mutant isolates showed low levels of resistance. The reduced sensitivity to pydiflumetofen resulted from the positive correlation of pydiflumetofen with the other four SDHI fungicides (i.e., boscalid, fluopyram, isopyrazam, and benzovindiflupyr). These results suggest that pydiflumetofen provides effective control for the management of gray mold but must be used with caution.

Keywords: baseline sensitivity, boscalid, *Botrytis cinerea*, pydiflumetofen

Gray mold, caused by *Botrytis cinerea* Pers.:Fr. (teleomorph *Botryotinia fuckeliana*), is a common disease of vegetables and fruits (Zheng et al. 2015), and its management mainly relies on chemical control (Fan et al. 2017). However, *B. cinerea* is known as a classical “high-risk” pathogen because of its extensive host range and high genetic variability (He et al. 2018a). Indeed, *B. cinerea* has developed resistance to many categories of fungicide, such as benzimidazoles, anilinopyrimidines, and dicarboximides. This makes it very difficult to effectively manage gray mold, and it is urgent to find a highly active rotational fungicide.

The action of succinate dehydrogenase inhibitors (SDHIs) on mitochondrial succinate dehydrogenase (SDH) complex II blocks cellular energy transfer, thereby inhibiting the development of pathogens in the field (Fraaije et al. 2012). As a result of its broad spectrum against fungi, this class of compounds has developed rapidly since the advent of boscalid (Lalève et al. 2014). To date, 18 SDHI fungicides, such as fluopyram, isopyrazam, and benzovindiflupyr, have been introduced to the market to control numerous phytopathogenic fungi (Hu et al. 2016). However, only boscalid and penthiopyrad have been registered in China for controlling gray mold (China

Pesticide Information Software). Pydiflumetofen is a new SDHI fungicide developed by Syngenta (Buxton et al. 2016); to date, there are numerous reports of the effective controls of pydiflumetofen against *Fusarium asiaticum* and *Sclerotinia sclerotiorum* (Duan et al. 2019; Hou et al. 2017). However, no detailed information was reported on the activity of pydiflumetofen on *B. cinerea*. Furthermore, the frequent use of SDHI fungicides against *B. cinerea* has led to serious resistance worldwide (Bardas et al. 2010; Kim and Xiao 2010; Wang et al. 2015). Previous studies have found that the resistance of *B. cinerea* to SDHIs is correlated with mutations at amino acids 225 (P225L/F/T), 230 (N230I), and 272 (H272Y/R/L) of the *SdhB* subunit (Yin et al. 2011), and different mutant isolates exhibit different degrees of resistance to a variety of SDHI fungicides such as fluopyram, isopyrazam, and fluxapyroxad (Veloukas et al. 2013). Whether the activity of pydiflumetofen on *B. cinerea* is affected by current SDHI-resistant isolates is unknown.

Therefore, the objectives of this study were as follows: (i) to assess the sensitivity of 291 *B. cinerea* isolates to pydiflumetofen collected in Shandong Province from 2017 to 2019, (ii) to evaluate the efficacy of pydiflumetofen on detached tomato fruits inoculated with SDHI-sensitive and SDH-resistant isolates, (iii) to determine the correlation between pydiflumetofen and the other four SDHI fungicides (i.e., boscalid, fluopyram, isopyrazam, and benzovindiflupyr), and (iv) to examine the efficacy of pydiflumetofen on cucumber leaves against gray mold in a greenhouse.

Materials and Methods

Fungicides. Technical-grade pydiflumetofen (95% active ingredient [a.i.]; Syngenta China Investment Co., Ltd.), fluopyram (96% a.i.; Bayer Crop Science), benzovindiflupyr (97% a.i.; Syngenta China Investment Co., Ltd.), boscalid (96% a.i.; Zhejiang Heben Technology Co., Ltd.), and isopyrazam (92% a.i.; Syngenta Switzerland Crop Protection Co., Ltd.) were dissolved in methanol to prepare stock solutions, which were stored at 4°C in the dark for use

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in vitro sensitivity assays. Formulated pydiflumetofen (200 g/liter SC; Syngenta China Investment Co., Ltd.) and boscalid (Cantus 50% WG; BASF China Co., Ltd.) were used in the detached tomato assay and greenhouse experiment.

Fungal isolates and sequencing of the *SdhB* gene. Diseased vegetables with characteristics of *B. cinerea* infection were collected from 10 regions in the Shandong Province of China. A total of 291 *B. cinerea* isolates (91 in 2017, 93 in 2018, and 107 in 2019) were isolated and identified by strict procedures (morphology, genealogical, biochemical, molecular, etc.) according to a previously described method (Fernández-Ortuño et al. 2012). These isolates were stored at 4°C. According to the method of Leroux et al. (2010), the DNA of the 291 isolates was extracted using the Fungal DNA Mini Kit (Omega Bio-Tek, Doraville, GA) and sequenced on the *SdhB* gene to identify the genetic background. Forward and reverse PCR primers (5'-CCACTCTCCATAATGGCTGCTCTCCGC-3' and 5'-CTCATCAAGCCCCCTCATTGATATC-3', respectively) were designed to amplify gene encoding for the *SdhB* subunit (GenBank accession KR866382.1). PCR amplifications were performed in a 50-μl reaction mixture containing 5 μl of 10× EasyTaq DNA polymerase buffer, 4 μl of dNTP, 2 μl of DNA template, 1 μl of each primer, 0.5 μl of EasyTaq DNA polymerase, and 36.5 μl of ddH₂O. Amplification conditions consisted of 94°C for 90 s; followed by 30 cycles at 94°C for 20 s, 56°C for 20 s, and 72°C for 60 s; and a final extension at 72°C for 5 min. The PCR products were visualized by electrophoresis on a 1.0% agarose gel run in 1× TAE buffer and then purified using the EasyPure Quick Gel Extraction Kit (TransGen Biotech, China). The purified products were cloned into the pGEM-T vector (Huayueyang Biotechnology Co., Ltd., Beijing, China) and sequenced by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. (China). DNAMAN (version 6.0; Lynnon Biosoft, Quebec, Canada) was used to compare the sequences of the amplified fragments.

Sensitivity of *B. cinerea* to pydiflumetofen in vitro. According to the previously described method (Song et al. 2016), the sensitivity of *B. cinerea* to pydiflumetofen was determined by spore germination inhibition assays. The spore germination inhibition assay was performed on yeast peptone acetate (YBA) medium (10 g of yeast extract, 10 g of Bacto peptone, 20 g of sodium acetate, and 15 g of agar per liter of distilled water) with final concentrations of pydiflumetofen at 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, and 1 mg/liter. Plates amended with equal volumes of methanol were used as controls. The EC₅₀ value (the fungicide concentration resulting in a 50% inhibition compared with that of the control) was calculated for each isolate. Three replicate plates were used for each treatment, and all experiments were performed three times. Resistance factors (RFs) were calculated by dividing the EC₅₀ value of resistant isolates by the mean EC₅₀ value of sensitive isolates. According to Veloukas et al. (2011), the following criteria were used for isolate classification: 10 > RFs > 2, isolate with low resistance; 100 > RFs > 10, isolate with moderate resistance; and RFs > 100, isolate with high resistance.

Efficacy of pydiflumetofen on detached tomatoes inoculated with SDHI-sensitive and SDHI-resistant isolates. According to the method of Veloukas et al. (2013), 15 *B. cinerea* isolates were selected to determine the efficacy of pydiflumetofen on detached tomato fruits. Three isolates of the sensitive, P225F, N230I, H272Y, and H272R mutants were used (Supplementary Table S1). The most commonly used SDHI fungicide, boscalid, was used as a control fungicide. Tomato fruits with similar size without any fungicide treatment were collected from a greenhouse. The surfaces of the tomato fruits were sterilized with 0.5% sodium hypochlorite for 1 min, rinsed three times with sterile distilled water, and then air dried. The surface-sterilized tomato fruits were sprayed with pydiflumetofen at 100, 200, and 300 mg/liter, as well as the control fungicide boscalid at 300 mg/liter, using a hand-held sprayer (Xinmeir Co., Ltd.). The control fruits were sprayed with sterile distilled water. The spores of *B. cinerea* were obtained from 7-day-old colonies. Conidial suspensions were prepared with sterile water to a concentration of 1 × 10⁵ spores/ml. A sterile fine needle was used to create a wound near the equator of the fruit, and 20 μl of the conidia suspension was inoculated into the wound. All of the treated fruits were kept for

3 days at 25°C and 80% relative humidity. The lesion size was then determined by measuring their two diameters at right angles to each other. Disease control efficacy was calculated as follows:

$$\text{Efficacy}(\%) = \frac{(\text{Lesion diameter in the water control} - \text{Lesion diameter in the treatment}) / \text{Lesion diameter in the water control} \times 100}$$

Three tomato fruits were used for each treatment, and all experiments were performed three times.

Correlation between pydiflumetofen and the other four SDHI fungicides. Seventy-two *B. cinerea* isolates that showed different levels of sensitivity to boscalid, fluopyram, isopyrazam, and benzovindiflupyr were selected from the isolates collected in 2018. The sensitivity of the 72 isolates to five SDHI fungicides (i.e., pydiflumetofen, boscalid, fluopyram, isopyrazam, and benzovindiflupyr) was determined by spore germination inhibition assays on YBA medium. Plates amended with equal volumes of methanol were used as controls. The concentrations of each fungicide were as follows: boscalid at 0, 0.05, 0.1, 0.5, 1, 5, 10, and 50 mg/liter; fluopyram at 0, 0.005, 0.01, 0.05, 0.1, 0.5, 1, and 5 mg/liter; isopyrazam at 0, 0.005, 0.01, 0.05, 0.1, 0.5, 1, and 5 mg/liter; and benzovindiflupyr at 0, 0.005, 0.01, 0.05, 0.1, 0.5, 1, and 5 mg/liter. Three replicate plates were used for each treatment, and all experiments were performed three times.

Greenhouse experiment. A greenhouse experiment was conducted in December 2018 on cucumber plants (cultivar Xintai Mici) in the village of Yuesihan (36.77°N, 118.69°E), Shouguang City, Shandong Province, China. *B. cinerea* infection was prevalent in this greenhouse. Five spray application treatments were used: (i) water control; (ii, iii, and iv) pydiflumetofen at 100, 200, and 300 mg/liter; and (v) control fungicide boscalid at 400 mg/liter. All treatments were laid out in a randomized complete block design with three replications. Before spray application, 100 leaves with or without disease symptoms in each plot were marked with red strings, and the disease index was recorded. Spraying was performed twice at a 7-day interval. The efficacy and the disease index of the leaves were calculated 7 days after the last application according to a previously described method (Song et al. 2016).

Data analysis. EC₅₀ values were calculated via regression of the percent relative growth against the log₁₀ values of the fungicide concentrations. The sensitivity of *B. cinerea* isolates to pydiflumetofen was plotted against their sensitivity to fluopyram, benzovindiflupyr, isopyrazam, and boscalid. The EC₅₀ values were transformed into log₁₀ values, and correlations of *B. cinerea* sensitivities to pydiflumetofen and the other four SDHI fungicides were evaluated by Pearson correlation coefficients (*r*). All of the data are represented as the mean ± SE. Analysis of variance was carried out using Fisher's least significant difference test (SPSS version 13.0 for Windows) to determine statistically significant differences among the treatments (*P* = 0.05). The efficacy of the fungicides against gray mold was arcsine transformed prior to analysis.

Results

Sensitivity of *B. cinerea* to pydiflumetofen in vitro. Pydiflumetofen exhibited strong inhibitory activity against *B. cinerea*, and there

Table 1. Sensitivity of *Botrytis cinerea* to pydiflumetofen based on spore germination

Year	Isolates (n)	EC ₅₀ (mg/liter)	
		Range	Mean ± SE ^z
2017	91	0.001–0.29	0.06 ± 0.01 a
2018	93	0.001–0.72	0.07 ± 0.02 a
2019	107	0.001–0.79	0.05 ± 0.02 a

^z Mean values followed by the same letter in the columns were not significantly different by Fisher's least significant difference test at *P* = 0.05. EC₅₀, fungicide concentration resulting in a 50% inhibition compared with that of the control.

was no difference in the sensitivity of *B. cinerea* to pydiflumetofen in 3 years (Table 1). The mean EC₅₀ values of pydiflumetofen for spore germination in 2017, 2018, and 2019 were 0.06 ± 0.01, 0.07 ± 0.02, and 0.05 ± 0.02 mg/liter, respectively. The mean EC₅₀ values of *B. cinerea* isolates collected from Laiwu and Liaocheng were significantly higher than those in the other eight regions (Supplementary Table S2). Among six crops, the mean EC₅₀ value of *B. cinerea* isolates collected from eggplant was the highest (Supplementary Table S3). The baseline sensitivity of *B. cinerea* to pydiflumetofen was established using 234 single-spore isolates without any mutation. The frequency distribution of the EC₅₀ values fit a unimodal curve (Fig. 1), with a mean value of 0.03 ± 0.003 mg/liter.

A total of 57 isolates with point mutations were detected from 291 isolates. The isolates carrying P225F, N230I, H272Y, and H272R mutations in the *SdhB* subunit were associated with the less sensitivity of *B. cinerea* to SDHIs. The mean EC₅₀ value of pydiflumetofen for the isolates with P225F, N230I, H272Y, and H272R mutation was 0.31 ± 0.09, 0.07 ± 0.02, 0.29 ± 0.05, and 0.08 ± 0.02 mg/liter, respectively (Table 2). The RFs of P225F and H272Y mutant isolates were the highest among the four mutation types, and they showed low to moderate levels of resistance to pydiflumetofen. The N230I and H272R mutant isolates showed low levels of resistance.

Efficacy of pydiflumetofen on detached tomato fruits. There was no significant difference in the efficacy of pydiflumetofen and boscalid against the sensitive isolates, and the efficacy of

pydiflumetofen and boscalid at 300 mg/liter was 91.9 and 91.0%, respectively (Fig. 2B). However, the efficacy of both fungicides was affected by the mutation types of the inoculated isolates (i.e., P225F, N230I, H272Y, and H272R). Comparatively, pydiflumetofen at 200 and 300 mg/liter provided significantly higher efficacy than boscalid at 300 mg/liter for mutant isolates (Fig. 2). Among four mutation types, P225F was the most difficult to control (Fig. 2C).

Correlation between pydiflumetofen and the other four SDHI fungicides. There was a positive correlation between pydiflumetofen and the other four SDHI fungicides (i.e., boscalid, fluopyram, isopyrazam, and benzovindiflupyr) (Fig. 3). The correlation coefficient between pydiflumetofen and isopyrazam was the largest, with an *r* value of 0.62 (*P* < 0.05).

Greenhouse experiment. Although there was no significant difference in the efficacy of pydiflumetofen and boscalid against cucumber gray mold, the efficacy of pydiflumetofen at 300 mg/liter was much higher than that of boscalid at 400 mg/liter. The efficacy of pydiflumetofen at 300 mg/liter for leaves was 80.9%, whereas that of boscalid at 400 mg/liter was 42.7% (Fig. 4).

Discussion

Currently, SDHI fungicides have become a research hotspot because of their wide control spectrum of diseases and unique mechanism of action (Xiong et al. 2017). Pydiflumetofen is a novel SDHI fungicide developed by Syngenta, and its activity on *B. cinerea* is largely unknown. It is well known that a positive correlation usually exists between SDHI fungicides (Avenot and Michailides 2010). Therefore, it is not only important to understand the activity of pydiflumetofen on SDHI-sensitive isolates of *B. cinerea*, but it is also essential to evaluate the efficacy of pydiflumetofen on SDHI-resistant isolates.

Consistent with the reported sensitivity of *B. cinerea* to other SDHI fungicides (He et al. 2018a; Song et al. 2016; Veloukas and Karaoglanidis 2012), the new SDHI fungicide pydiflumetofen had strong inhibitory activity on spore germination of *B. cinerea*. This study found that there was no difference in the sensitivity of *B. cinerea* to pydiflumetofen among the 3 years (2017, 2018, and 2019). This finding indicated that the inhibitory activity of pydiflumetofen against *B. cinerea* was very stable. According to China Pesticide Information Software, we found that conventional fungicides such as carbendazim, boscalid, procymidone, and iprodione accounted for a large percentage of the registered fungicides for controlling gray mold. However, *B. cinerea* has been severely resistant to benzimidazoles and dicarboximides (Liu et al. 2016; Rupp et al. 2017). Because of the unique mechanism of action, pydiflumetofen shows no cross-resistance with conventional fungicides such as benzimidazoles and dicarboximides (Duan et al. 2019). Therefore, we believed that pydiflumetofen has a potential application for management of benzimidazole- and dicarboximide-resistant isolates. What is more, pydiflumetofen has great control for SDHI-resistant isolates of *B. cinerea*. It is well known that boscalid is the most commonly used SDHI fungicide for controlling gray mold in China. Frequent use of boscalid has led to the emergence of resistant isolates of *B. cinerea* in the field (Gutiérrez-Alonso et al. 2017). We found that the isolates carrying P225F, N230I, H272Y, and H272R mutations in the *SdhB* subunit were responsible for the less sensitivity of *B. cinerea* to boscalid in Shandong Province. These resistant isolates have led to the failure of boscalid for controlling gray mold at registered concentrations, which was confirmed by the detached tomato assay in the current study. We also found that the efficacy of pydiflumetofen was superior to boscalid against gray mold in both the detached tomato assay and the greenhouse experiment. This finding suggested that pydiflumetofen could provide effective management of the current resistance to SDHI fungicides. Previous studies found that the protective effect of pydiflumetofen is better than its curative effect (Duan et al. 2019), which is consistent with other SDHI fungicides (He et al. 2018a; Song et al. 2016; Veloukas and Karaoglanidis 2012). Therefore, pydiflumetofen is recommended for use in the initial stage of gray mold occurrence.

It is important to perform resistance risk assessment of new fungicides before they enter the market. Therefore, baseline sensitivity of pathogenic fungi to new fungicides should be established for

Fig. 1. Frequency distributions of EC₅₀ values for pydiflumetofen against 234 *Botrytis cinerea* isolates collected in Shandong Province of China based on spore germination. EC₅₀, fungicide concentration resulting in a 50% inhibition compared with that of the control.

Table 2. Sensitivity of *Botrytis cinerea* with four mutation types to pydiflumetofen based on spore germination

Mutation type	Isolates (n)	EC ₅₀ (mg/liter)		
		Range	Mean ± SE ^y	Resistance factor ^z
P225F	9	0.05–0.79	0.31 ± 0.09 a	1.6–26.3
H272Y	16	0.06–0.73	0.29 ± 0.05 a	2.0–24.3
N230I	13	0.02–0.19	0.07 ± 0.02 b	0.6–6.3
H272R	19	0.01–0.39	0.08 ± 0.02 b	0.3–13.0

^y Mean values followed by the same letter in the columns were not significantly different by Fisher's least significant difference test at *P* = 0.05. EC₅₀, fungicide concentration resulting in a 50% inhibition compared with that of the control.

^z Resistance factors were calculated by dividing the EC₅₀ value of resistant isolates by the mean EC₅₀ value of sensitive isolates.

Fig. 2. Efficacy of pydiflumetofen on detached tomatoes inoculated with different phenotypes of *Botrytis cinerea*. **A**, visible symptoms; **B**, sensitive; **C**, P225F; **D**, N230I; **E**, H272R; and **F**, H272Y). Mean values followed by the same letter in the columns were not significantly different by Fisher's least significant difference test at $P = 0.05$.

Fig. 3. Correlation between pydiflumetofen and **A**, fluopyram, **B**, boscalid, **C**, benzovindiflupyr, and **D**, isopyrazam. EC_{50} , fungicide concentration resulting in a 50% inhibition compared with that of the control.

subsequent monitoring of fungal resistance (Huang et al. 2019). After establishing the sensitivity baseline of *B. cinerea* to pydiflumetofen, we evaluated the resistance level of *B. cinerea* isolates with four mutation types to pydiflumetofen. Many studies reported that mutation types P225F and N230I are responsible for the resistance of *B. cinerea* to boscalid, fluopyram, and isopyrazam; H272R and H272Y mutant isolates are moderately resistant to boscalid and have low resistance to isopyrazam but are sensitive to fluopyram (Lalève et al. 2014; Veloukas et al. 2013). We found that P225F and H272Y mutant isolates showed low to moderate levels of resistance to pydiflumetofen, and H272R and N230I mutant isolates showed a low level of resistance. In the current study, we first proved that the reduced sensitivity of *B. cinerea* with four mutation types to pydiflumetofen was mainly attributable to the positive correlation of pydiflumetofen with the other four SDHI fungicides (i.e., boscalid, fluopyram, isopyrazam, and benzovindiflupyr). As we know, once the structure of the target protein changes, its binding energy to the fungicide compound generally decreases, thereby resulting in fungicide resistance (Kretschmer et al. 2009). Previous studies reported that pyrazole is more potent than pyridine in combination with the target protein, and introducing the fluorine atom into the compound structure increases the binding energy of the compound to the target protein (Fraaije et al. 2012; Scalliet et al. 2012). Our results showed that *B. cinerea* isolates with four mutation types were more sensitive to pydiflumetofen than boscalid. We speculate that maybe the structural characteristics of pyrazole and fluorine atoms on pydiflumetofen (Supplementary Fig. S1) result in the sensitivity difference between pydiflumetofen and boscalid. In the current study, the P225F mutation was less sensitive to pydiflumetofen than other mutation types, and the resistance frequency of the P255F mutation was highly detected in the cities of Laiwu and Liaocheng in Shandong Province. Therefore, although pydiflumetofen could effectively manage the current resistance of SDHI fungicides, it still should be used with caution in specific areas.

In conclusion, pydiflumetofen is currently effective for controlling gray mold, and it can be used as a substitute for boscalid to manage gray mold in the field. However, current SDHI-resistant isolates of *B. cinerea* showed reduced sensitivity to pydiflumetofen, although the resistance to pydiflumetofen was only at low to moderate levels. SDHI fungicides are marked with a medium to high resistance risk, which makes us aware of the importance of delaying the development of resistance (Fungicide Resistance Action Committee 2018). In the future, the sensitivity of *B. cinerea* to pydiflumetofen should be continuously monitored to effectively adjust the application schedule. In addition, precise application techniques can decrease the selective pressure of fungicides, thereby delaying the development of fungal resistance (He et al. 2018b). Therefore, future research should focus on exploring more efficient application techniques.

Fig. 4. Efficacy of pydiflumetofen and boscalid on cucumber leaves in the greenhouse experiment. Mean values followed by the same letter in the columns were not significantly different by Fisher's least significant difference test at $P = 0.05$.

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