Baseline Sensitivity and Toxic Action of the Sterol Demethylation Inhibitor Flusilazole Against *Botrytis cinerea*

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Abstract

In the present study, a total of 95 *Botrytis cinerea* single-spore strains collected from different hosts in Shaanxi Province of China were characterized for their sensitivity to the sterol demethylation inhibitor fungicide flusilazole. The effective concentration for 50% inhibition of mycelial growth (EC₅₀) of flusilazole ranged from 0.021 to 0.372 µg/ml, with an average value of 0.093 µg/ml. Cross-resistance between flusilazole and commonly used fungicides was not detected, and no flusilazole-resistant mutants were induced. Both on detached strawberry leaves and in greenhouse experiments, flusilazole was more effective than the commonly used fungicide carbendazim at reducing gray mold. After culture on PDA plates or detached strawberry leaves, no difference in sclerotia production or pathogenicity was detected between two strains, WG12 (most sensitive to flusilazole) and MX18 (least sensitive to flusilazole). After treatment with flusilazole, however, the two strains lost the

ability to produce sclerotia, and oxalic acid and ergosterol contents in mycelium decreased. Interestingly, the inhibition rate of ergosterol content in MX18 was significantly lower than that in WG12. Expression of *Cyp51*, *BcatrD*, and *Bcmfs1* genes all increased after treatment with flusilazole, especially the *Cyp51* and *BcatrD* genes. However, the expression of *Cyp51* gene or *BcatrD* gene in WG12 and MX18 were significantly different from each other after treatment with flusilazole. In addition, no point mutations in *Cyp51* gene were found in MX18. These data suggest flusilazole is a promising fungicide for resistance management of gray mold and also provided novel insights into understanding the resistance mechanism of flusilazole against plant pathogens.

Keywords: flusilazole, gray mold, baseline sensitivity, resistance mechanism

The necrotrophic filamentous fungus *Botrytis cinerea* Pers.:Fr., the causal agent of gray mold, is a highly devastating plant pathogen notorious for its wide host range and wide distribution (Elmer and Reglinski 2006; Rotolo et al. 2018; Sabrina et al. 2016; Williamson et al. 2007). More than 1,400 plant species, including important protein, oil, fiber, and horticultural crops, can be infected by *B. cinerea* in temperate and subtropical regions (Coertze and Holz 2002; Elad et al. 2015; Elmer and Michailides 2007; Van Kan et al. 2017). Leaves, shoots, bulbs, flowers, fruit, and root tubers at different growth stages are susceptible to *B. cinerea* infection. The pathogen not only reduces crop yields but also causes significant damage to the quality of the resulting produce (Amselem et al. 2011; Liu et al. 2019; Van Kan 2006). *B. cinerea* from the class Leotiomycetes, family Sclerotiniaceae, is a classical “high-risk” pathogen because it has a variety of modes of attack, diverse hosts as inoculum sources, and can survive as mycelia, conidia, and/or sclerotia for an extended period in crop debris (Myresiotis et al. 2007; Song et al. 2016; Williamson et al. 2007).

Currently control of gray mold caused by *B. cinerea* is dependent on application of synthetic agrochemicals. Several families of synthetic fungicides, such as benzimidazole fungicides, dicarboximide fungicides, anilinopyrimidine fungicides, succinate dehydrogenase inhibitor fungicides, and quinone outside inhibitor fungicides have been registered for use against gray mold worldwide (Rotolo et al.

2018; Sabrina et al. 2016). Unfortunately, failures to control the disease have been reported in many countries due to the development of resistance to these fungicides (Bardas et al. 2010; Cong et al. 2018; Myresiotis et al. 2008; Song et al. 2016; Sun et al. 2010). Thus, it is necessary that alternative candidates belonging to different chemical groups with no cross-resistance with fungicides already in use are explored for the management of fungicide resistance in controlling gray mold.

Flusilazole belongs to a novel chemical class of triazole compounds, a chemical group within the class of sterol demethylation inhibitor (DMI) with broad spectrum and high efficacies against a wide range of plant diseases. DMI fungicides target the C-14 demethylation of 24-methylene dihydrolanosterol, a precursor of fungal sterol biosynthesis (Becher and Wirsal 2012; Diaz-Trujillo et al. 2018; Romero and Sutton 1997). Previous studies have demonstrated that the LD₅₀ of flusilazole in rat is 674 mg/kg, indicating slight toxicity to mammals. Flusilazole was widely used in agriculture, horticulture, and viticulture for control of a variety of plant pathogenic fungi for several decades worldwide (Ozakca and Silah 2013). In China, flusilazole has been registered for control of a wide range of plant diseases such as apple ring rot (*Botryosphaeria dothidea* [Moug.] Ces. & De Not), apple scab (*Venturia inaequalis* Cooke [Wint.]), powdery mildew on cucumber (*Erysiphe cichoracearum* DC ex Mecat), wheat leaf rust (*Puccinia triticina* Eriks.) and stripe rust (*Puccinia striiformis* f. sp. *tritici*), barley leaf spot (*Bipolaris sorokiniana* [Sacc.] Shoemaker), anthracnose on apple (*Colletotrichum gloeosporioides* [Penz.] Penz. & Sacc.), tomato leaf mold (*Fulvia fulva* [Cooke] Ciferri), and grapevine white rot (*Coniothyrium diplodiella* [Speg.] Sacc.) (Lu et al. 2018). However, the fungicide has not been registered for control of gray mold on vegetables or fruits, and thus the pathogen has not been exposed to flusilazole (China Pesticide Information Network: <http://www.icama.org.cn/>). Due to the single action site of this fungicide, flusilazole resistance has been observed in several plant pathogens, such as *V. inaequalis* (Kunz et al. 1997), *Uncinula necator* (Schwein.) Burr. (Halleen et al. 2000), *F. fulva* (Ye et al. 2012), and *B. dothidea* (Liu et al. 2013). However, the resistance mechanism of pathogens to flusilazole is still not fully understood.

Establishment of baseline sensitivity is a significant step before registration of a novel fungicide for control of a plant disease.

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Therefore, the present study aimed to (i) establish the baseline sensitivity of *B. cinerea* to flusilazole; (ii) determine the cross-resistance pattern between flusilazole and other commonly used fungicides; (iii) evaluate the efficacy of flusilazole against *B. cinerea*; (iv) compare the biochemical characteristics of the strains identified with low or high flusilazole sensitivity *in vitro*; and (v) explore the potential resistance mechanism of *B. cinerea* to flusilazole.

Materials and Methods

***B. cinerea* strains and fungicides.** A total of 95 single-spore strains of *B. cinerea* were collected from diseased fruits of grape and strawberry (57 strains were collected from vineyards, and 38 strains were collected from strawberry greenhouses) with typical gray mold symptoms and obvious spores in Shaanxi Province in 2017 and 2018. All 95 *B. cinerea* strains had previously been exposed to carbendazim, procymidone, or pyrimethanil for many years but had not been exposed to flusilazole. Mycelium cut from pure cultures was transferred and stored on PDA slants at 4°C in the dark.

Technical-grade carbendazim (97.0%; Shenyang Academy of Chemistry and Industry, Shenyang, China) was dissolved in hydrochloric acid (HCl, 0.1 mol/liter) to produce a 10 mg/ml stock solution. Procymidone (98.5%; Sumitomo Chemical Company, Kyoto, Japan), pyrimethanil (95.2%; Fengdeng Chemical Factory, Jiangsu, China), and flusilazole (95.0%; Shaanxi Heng Run Chemical Industry Co., Shaanxi, China) were dissolved in methanol to obtain stock solutions at 10 mg/ml, respectively.

Baseline sensitivity to flusilazole *in vitro*. Sensitivity tests of 95 *B. cinerea* strains to flusilazole were determined by a mycelial growth inhibition method. Briefly, mycelial plugs (5 mm in diameter) cut from the margin of 3-day-old colonies were transferred to the center of potato dextrose agar (PDA) plates containing 0, 0.03125, 0.0625, 0.125, 0.25, 0.5, or 1 µg/ml flusilazole, respectively. Each colony diameter was measured twice at right angles after 3 days of incubation at 25°C in the dark. The effective concentration for 50% inhibition of mycelial growth (EC₅₀) value for each strain was calculated by regressing the relative inhibition of mycelial growth against the log₁₀ flusilazole concentration (Wang et al. 2019). The experiment was conducted twice with three plates per concentration.

Cross-resistance analysis. Cross-resistance between flusilazole and other fungicides was conducted using 12 *B. cinerea* strains that varied in their sensitivity to flusilazole (Table 1). Single discriminatory concentration testing was used to test their sensitivity to carbendazim or procymidone. Strains were designated resistant if they could grow on the PDA plates amended with 5 µg/ml of carbendazim or procymidone (Liu et al. 2016). To test their sensitivity to pyrimethanil, the fungicide concentrations were 0, 0.063, 0.125, 0.25, 0.5, and 1 µg/ml used for the pyrimethanil-sensitive strains, and 0, 3.125, 6.25, 12.5, 25, 50, and 100 µg/ml used for the pyrimethanil-resistant strains. Each colony diameter was measured twice at right angles after 3 days of incubation at 25°C in the dark. Cross-resistance between flusilazole and carbendazim, procymidone, or pyrimethanil was analyzed based on their EC₅₀ values. The experiment was conducted twice with three plates per concentration.

Efficacy of flusilazole against *B. cinerea* on detached tissue. Two strains, DZ10 (resistant to carbendazim) and MX18 (least sensitive to flusilazole and sensitive to carbendazim), which were both collected from strawberry, were selected for a flusilazole efficacy test on detached strawberry leaves. Briefly, strawberry leaves (Hongyan) with similar growth stage were soaked for 15 s in water, carbendazim at 200 µg/ml, or flusilazole at 50, 100, or 200 µg/ml, respectively. After being air dried, each strawberry leaf was punctured once (avoiding the major vein of the leaves) with a sterilized needle and inoculated with an inverted mycelial plug (0.5 cm diameter) cut from the edge of 3-day-old colonies of the strains described above. There was one mycelial plug per leaf. Inoculated leaves were placed in plastic trays (80 × 60 × 10 cm) lined with moist filter paper. The plastic trays were placed in a growth chamber (25°C, 12-h photoperiod, and 85% relative humidity). An energy saving lamp (FSL YDW40-HB, Guangzhou, China) with three primary colors 6,500 K was used in

the growth chamber (KeMai KM-1000, Ningbo, China). After 3 days, the diameter of the lesion that developed on each leaf was measured twice in two perpendicular directions and averaged. The control efficacy was calculated by the following formula: control efficacy (%) = [(lesion diameter of control – lesion diameter of treatment) / (lesion diameter of control – 0.5 cm)] × 100% (Wang et al. 2019; Zhang et al. 2018). The experiment was repeated three times with six leaves per concentration.

Efficacy of flusilazole against *B. cinerea* in the straw berry greenhouse. This experiment was performed in two strawberry greenhouses naturally infested with *B. cinerea* in Yangling (Located in Shaanxi Province of China) in late February 2018. In each greenhouse, five treatments with three replicate plots (20 m² per plot) per treatment were completely randomized in design. The treatments were (i) water control, 750 liters/ha; (ii) carbendazim (50% WP), 375 g of active ingredient (a.i.)/ha; (iii) flusilazole (400 g/liter EC), 375 g of a.i./ha; (iv) flusilazole, 187.5 g of a.i./ha; and (v) flusilazole, 93.75 g of a.i./ha. Fungicides were applied with a Jacto Heavy-Duty HD400 sprayer (Agrolex, Singapore) at early flowering for the first spray. Fungicides were applied three times with 7 days between sprays. No other fungicides were applied before, and the treatments were given in accordance with standard farm practices. Disease incidence was surveyed 2 weeks after the last spray by examining 50 randomly selected (five-point sampling method) strawberry fruits in each plot for the symptoms of gray mold. The disease severity was divided according to the percentage of the lesion area as a percentage of the whole fruit as follows: 0, no symptoms; 1, <5%; 3, 6 to 10%; 5, 11 to 20%; 7, 21 to 50%; and 9, >50%. Disease index and control efficacy were calculated as follows: disease index = $\sum$ (disease severity × number of diseased fruit) / (the highest disease severity × number of total surveyed fruit) × 100; control efficacy (%) = (disease index of control – disease index of treatment) / disease index of control × 100 (Gu et al. 2017).

Effect of flusilazole on sclerotia production and oxalic acid content. Sclerotia production between two strains, WG12 (collected from grape, most sensitive to flusilazole) and MX18 (collected from strawberry, least sensitive to flusilazole), was compared. Mycelial plugs cut from the leading edge of 3-day-old colonies were transferred mycelia-side down onto PDA plates amended with flusilazole at their EC₅₀ values. Plates without flusilazole were used as the non-treated control. After the PDA plates had been incubated at 25°C in darkness for 4 weeks, the number of sclerotia in each plate was recorded. The experiment was repeated three times with three plates per treatment.

Oxalic acid content was determined according to a previous study with minor modifications (Wang et al. 2019). Under acidic conditions, iron (III) and oxalate form a complex that discolors the complex of iron (III) and sulfosalicylic acid. Therefore, the content of oxalate could be measured indirectly by determining the intensity

Table 1. Analysis of cross-resistance

Strains	Sensitivity to		EC ₅₀ (µg/ml) for	
	Carbendazim	Procymidone	Pyrimethanil	Flusilazole
YL9	S ^y	S	0.098 c ^z	0.109 c
YL27	S	R	0.107 c	0.172 b
MX4	S	S	0.193 c	0.180 b
RG6	R	S	0.154 c	0.101 c
RG17	R	S	0.178 c	0.082 c
MX18	S	S	0.156 c	0.372 a
DZ10	R	R	0.109 c	0.093 c
ZZ21	S	R	0.167 c	0.154 b
WG12	S	S	0.153 c	0.021 d
DZ22	R	S	11.52 b	0.099 c
WQ11	S	S	21.01 a	0.187 b
DZ5	R	S	17.83 a	0.092 c

^y S and R represent sensitivity and resistance to fungicide, respectively.

^z Values in columns followed by the same letters are not significantly different according to Fisher's protected LSD test ($P = 0.05$).

of the color of the complex at 510 nm with a spectrophotometer (Shimadzu UV2600, Kyoto, Japan). To establish a standard curve for oxalate, 10 ml of HCl-KCl buffer (KCl 50 mM, pH = 2), 1 ml of FeCl₃ solution (0.5 mg/ml), and 0.5 ml of sulfosalicylic acid solution (5 mg/ml) were added to a 50-ml centrifuge tube. Different volumes (0, 0.1, 0.2, 0.4, or 0.8 ml) of sodium oxalate solution (2 mg/ml) were then added, and the volume in each of the centrifuge tubes was brought up to 25 ml using double-distilled water. The centrifuge tubes were vortexed for 10 s and incubated at 25°C for 30 min. Double-distilled water was used as a control. The absorbance was measured at 510 nm with a microplate reader (Infinite M200pro, NanoQuant, Salzburg, Austria). Finally, the standard curve was established by plotting absorbance against sodium oxalate concentration.

To determine the oxalic acid content in WG12 and MX18, mycelial plugs cut from the edge of 3-day-old colonies were transferred into 250-ml flasks (five plugs per flask) containing 100 ml of potato dextrose broth (PDA without agar) that was amended with flusilazole at each strain's respective EC₅₀ values. Flasks without flusilazole were used as a control. After the flasks were shaken on a rotary shaker (ZHWY-2112B, Shanghai, China) at 175 rpm and 25°C in the dark for 3 days, the contents were centrifuged at 1,500 rpm for 10 min. The absorbance of the supernatants was measured at 510 nm with a microplate reader. The oxalic acid content in the supernatants was calculated using the standard curve constructed (Supplementary Fig. S1). There were three flasks per treatment, and the experiment was repeated three times.

Pathogenicity evaluation. Strawberry leaves (cultivar Hongyan) or grapes (cultivar Zuijinxiang) with similar growth stage were rinsed three times with distilled water. After being air dried, each strawberry leaf was punctured once (avoiding the major vein of the leaves) with a sterilized needle, and each grape was punctured once (about 2 mm deep in the middle) with a sterilized needle. The wounded leaves or grapes were inoculated with inverted mycelial plugs (one mycelial plug per leaf or grape) cut from the edge of 3-day-old colonies of the two strains, WG12 or MX18. Inoculated leaves or grapes were placed in plastic trays (80 × 60 × 10 cm) lined with moist filter paper. Then the plastic trays were placed in a growth chamber (25°C, 12-h photoperiod, and 85% relative humidity). An energy-saving lamp (FSL YDW40-HB) with three primary colors 6,500 K was used in the growth chamber. After 3 days, the diameter of the lesion on each leaf or grape was measured twice in two perpendicular directions and photographed. The experiment was repeated three times with six leaves or grapes per strain.

Effect of flusilazole on ergosterol content. Ergosterol content in mycelia was analyzed by an HPLC method according to a previous study (Ren et al. 2015). First, a standard curve with standard ergosterol (Sigma, St. Louis, MO) was established by plotting peak area against ergosterol concentration. For each of the strains, WG12 or MX18, broth cultures were prepared as described above. After the flasks were shaken on a rotary shaker at 175 rpm and 25°C for 3 days in the dark, the mycelia were harvested, dried, and ground into powder, and 0.1 g of mycelia was transferred into 50-ml centrifuge tubes containing 20 ml of saponifier (10% KOH + methanol). The tube was placed in a water bath at 80°C for 2 h. The resultant solution was

extracted three times with petroleum ether and concentrated at 75 rpm and 40°C with a rotary evaporator (RE-3000A, Shanghai, China). The precipitate was dissolved in ethanol and diluted to 10 ml. Ergosterol content in the mycelia was detected by the HPLC method according to the standard curve.

HPLC detection conditions were as follows: Waters 600 and Bondapak-C₁₈ column (Waters Associates, Milford, MA) were used, and 90% methanol was used as the mobile phase. The flow speed was kept at 1 ml/min, the injection volume was 10 µl, the detection wavelength was 282 nm, and the column was kept at 25°C.

Effect of flusilazole on gene expression levels. Previous studies had demonstrated that the eburicol 14α-demethylase gene *Cyp51* (GenBank: AF279912.1), ATP-binding cassette (ABC) transporter gene *BcatrD* (GenBank: AJ272521.2), and the major facilitator superfamily (MFS) transporter gene *Bcmfs1* (GenBank: AF238225.2) were correlated with resistance to DMIs (Cools et al. 2010; Fraaije et al. 2007; Hayashi et al. 2001, 2002). Therefore, the expression levels of these genes in WG12 and MX18 were determined with quantitative real-time polymerase chain reaction (qRT-PCR) according to a previous study (Wang et al. 2019). Total RNA was extracted from the two strains. qRT-PCR was performed using a HiScript II One Step qRT-PCR SYBR Green Kit (Vazyme Biotech Co., Nanjing, China) on a CFX96 Touch Real-Time PCR detection system (Bio-Rad, Hercules, CA) according to the manufacturers' instructions. The following qRT-PCR parameters were used: reverse transcription at 95°C for 3 min; an initial preheat for 5 min at 95°C; followed by 40 cycles of denaturation at 95°C for 10 s and annealing and extension at 60°C for 30 s. The expression of the measured genes in each sample was normalized to actin gene expression and calculated using the 2^{-ΔΔCt} method (Livak and Schmittgen 2001). Data from three biological replicates were used to calculate the mean and standard deviation. The primers used for qRT-PCR are listed in Table 2. The experiment was conducted with three biological replicates and three technical replicates per treatment.

Statistical analysis. Data from repeated experiments were combined for analysis because variances between experiments were homogeneous. Two-way analysis of variance with repeat and concentration as two fixed factors was performed with the procedure of Univariate in general linear model in SPSS 22.0 (Statistical Package for the Social Science, SPSS, Chicago, IL). Normality analysis of the baseline sensitivity was conducted as follows: the EC₅₀ values of each of the 95 strains were used to calculate the kurtosis coefficient and skewness coefficient in Microsoft Excel; the Shapiro-Wilk test and Kolmogorov-Smirnov test were conducted by the statistical software Data Processing System (DPS, version 7.05; Hangzhou Ruitong Information Technology Co., Hangzhou, China). If significant differences were detected among the data for EC₅₀, flusilazole efficacy, sclerotia production, oxalic acid, ergosterol content, and gene expression, Fisher's least significant difference (LSD) test (*P* = 0.05) was used to compare means.

Results

Baseline sensitivity of *B. cinerea* to flusilazole. A total of 95 single-spore *B. cinerea* strains were tested for sensitivity to flusilazole on the basis of mycelial growth. The EC₅₀ values of the 95 wild-type strains of *B. cinerea* ranged from 0.021 to 0.372 µg/ml to flusilazole, with an average value of 0.093 µg/ml and a median value of 0.086 µg/ml. The EC₅₀ values of the most sensitive strain (WG12, collected from grape) and the least sensitive strain (MX18, collected from strawberry) were 0.021 and 0.372 µg/ml, respectively. The individual EC₅₀ values were separated by a variation factor (the highest EC₅₀ value divided by the lowest EC₅₀ value) of 17.71, and the frequency distribution of the EC₅₀ values was a unimodal curve (Fig. 1).

In addition, the EC₅₀ value of the 95 strains was used to calculate the kurtosis coefficient and skewness coefficient in Microsoft Excel. The skewness coefficient was 2.43 (>0), and the kurtosis was 9.90 (>3), which indicated that the EC₅₀ values did not meet the standard normal distribution. It was a severe right-skewed distribution with steep peaks, and the distribution had a long tail on the right.

Table 2. Primers used in this study

Primer	Sequence (5'-3')	Use
P1	GGGCAACGACTTTATTCTC	qRT-PCR ^z primers for the <i>Cyp51</i> gene
P2	CCATTGTATGATTGGGAC	
P3	GTCCACCTTTGCCGATTT	qRT-PCR primers for the <i>BcatrD</i> gene
P4	GTGTTAGCGAGACCCGTAG	
P5	TGGAGTGAACGCATAAAGG	qRT-PCR primers for the <i>Bcmfs1</i> gene
P6	ATCCGAGACAGAAGGAGAACC	
actin-F	CGTCTGGATTGGTGGTTCTATT	qRT-PCR primers for analysis of actin gene expression
actin-R	ACTCGTCGTACTCTTGCTTTG	

^z qRT-PCR = quantitative real-time polymerase chain reaction.

According to the Shapiro–Wilk test (W test), $W = 0.7990$ ($P = 0.00$), and according to the Kolmogorov–Smirnov test, $D = 0.0530$ ($P = 0.00$); thus, the sensitivity distribution did not conform to a normal distribution.

Cross-resistance analysis. Among the 12 representative strains tested, which varied in sensitivity to flusilazole, five were resistant to carbendazim, three were resistant to procymidone, and three were resistant to pyrimethanil. Their sensitivity to flusilazole differed from each other, and the EC_{50} values ranged from 0.021 to 0.372 $\mu\text{g/ml}$ (Table 1). No correlation was detected between sensitivity to

Fig. 1. Frequency distribution of EC_{50} values of 95 *Botrytis cinerea* strains to flusilazole measured by inhibition of mycelial growth on PDA amended with different concentrations of the fungicide.

Fig. 2. Efficacy of three rates of flusilazole and carbendazim against two strains of *Botrytis cinerea* on detached strawberry leaves. DZ10 = carbendazim-resistant strain; and MX18 = carbendazim-sensitive strain.

flusilazole and sensitivity to carbendazim, procymidone, or pyrimethanil, indicating no cross-resistance between flusilazole and the botryticides (carbendazim, procymidone, and pyrimethanil) already in commercial use.

Efficacy of flusilazole against *B. cinerea* on detached strawberry leaves. On detached strawberry leaves inoculated with either MX18 (carbendazim sensitive) or DZ10 (carbendazim resistant) and treated with water, large lesions were observed (Fig. 2, Table 3). Although lesions occurred on inoculated leaves treated with fungicide, they were smaller ($P < 0.05$). Fungicide efficacy of 79.2% was obtained by carbendazim at 200 $\mu\text{g/ml}$ when the leaves were inoculated with MX18, and efficacy was only 26.1% when leaves were inoculated with DZ10. Flusilazole provided significantly better efficacy than the reference fungicide (carbendazim) at the same concentration ($P < 0.05$). Over 95% efficacies were obtained when inoculated leaves were treated with flusilazole at 200 $\mu\text{g/ml}$ whether the leaves were inoculated with carbendazim-resistant or -sensitive strains. Moreover, flusilazole at 100 $\mu\text{g/ml}$ provided over 75% efficacy against the carbendazim-sensitive strain, which was equal to the efficacy of carbendazim at 200 $\mu\text{g/ml}$. The lowest rate of flusilazole, 50 $\mu\text{g/ml}$, showed over 45% efficacy against both the carbendazim-resistant and -sensitive strain.

Efficacy of flusilazole against *B. cinerea* in the strawberry greenhouse. In the two strawberry greenhouses, gray mold was severe, and the disease index of the water control plots was over 30% (Table 4). Flusilazole sprayed at 500 $\mu\text{g/ml}$ provided over 85% efficacy against *B. cinerea* on strawberry fruit, which was significantly greater than the efficacy obtained by carbendazim at the same concentration ($P < 0.05$). Over 70% efficacy was obtained by flusilazole at 250 $\mu\text{g/ml}$, although this was not different from the efficacy obtained by carbendazim at 500 $\mu\text{g/ml}$ ($P > 0.05$). Although nearly 50% efficacy was obtained when flusilazole was applied at 125 $\mu\text{g/ml}$, this rate was less effective than the standard rate of carbendazim ($P < 0.05$).

Effect of flusilazole on sclerotia production and oxalic acid content. After incubation on medium amended with no fungicide at 25°C in darkness for 4 weeks, many sclerotia were observed within the colony of each *B. cinerea* strain, WG12 and MX18 (Fig. 3). There was no significant difference between the number of sclerotia produced by either strain ($P > 0.05$) (Supplementary Table S1). When incubated on medium amended with flusilazole at each strain's respective EC_{50} value, no sclerotia were produced by either strain (Fig. 3). These data suggest flusilazole inhibits production of sclerotia.

Table 3. Efficacy of flusilazole against *Botrytis cinerea* on detached strawberry leaves

Treatment	Strawberry leaves inoculated with DZ10 ^y		Strawberry leaves inoculated with MX18 ^y	
	Lesion diameter (cm)	Control efficacy (%)	Lesion diameter (cm)	Control efficacy (%)
Flusilazole (50 $\mu\text{g/ml}$)	1.7 c ^z	47.7 c	1.7 b	50.0 c
Flusilazole (100 $\mu\text{g/ml}$)	1.0 d	78.3 b	1.0 c	79.2 b
Flusilazole (200 $\mu\text{g/ml}$)	0.6 e	95.7 a	0.6 d	95.8 a
Carbendazim (200 $\mu\text{g/ml}$)	2.2 b	26.1 d	1.0 c	79.2 b
Water control	2.8 a	...	2.9 a	...

^y DZ10 is resistant to carbendazim, and MX18 is sensitive to carbendazim.

^z Values in columns followed by the same letters are not significantly different according to Fisher's protected LSD test ($P = 0.05$).

Table 4. Control of gray mold in the strawberry greenhouse by carbendazim and flusilazole

Treatment	Dosage (g of a.i./ha)	Active ingredient ($\mu\text{g/ml}$)	Disease index	Disease control (%)
50% Carbendazim (WP)	375.0	500.0	10.3 c ^z	69.1 b
400 g/liter of Flusilazole (EC)	93.8	125.0	16.9 b	49.2 c
400 g/liter of Flusilazole (EC)	187.5	250.0	9.4 dc	71.8 b
400 g/liter of Flusilazole (EC)	375.0	500.0	4.5 d	86.5 a
Water	...	...	33.3 a	...

^z Data are the means of two strawberry greenhouses. Mean values with the same letters within the same column are not significantly different ($P = 0.05$, Fisher's LSD).

As shown in Figure 4, there was no significant difference in oxalic acid content between the two strains, WG12 and MX18, and oxalic acid content significantly decreased when each strain was treated with flusilazole at their respective EC_{50} values ($P < 0.05$).

Pathogenicity evaluation. Although the two strains tested varied in their sensitivity to flusilazole, they did not differ in pathogenicity on either detached strawberry leaves or grapes ($P > 0.05$) (Fig. 5, Supplementary Figs. S2 and S3). This indicated that the two strains have similar pathogenicity, and sensitivity to flusilazole could not affect pathogenicity.

Effect of flusilazole on ergosterol content. Ergosterol content in mycelia of each strain significantly decreased after treatment with flusilazole at each strain's respective EC_{50} values ($P < 0.05$) (Fig. 6). Interestingly, the content of ergosterol was reduced 44.8% in WG12 when it was grown on medium amended with flusilazole compared with the control with no flusilazole, which is nearly twofold more than that of MX18 (23.2%).

Effect of flusilazole on gene expression levels. Expression of *Cyp51*, *BcatrD*, and *Bcmfs1* genes in WG12 and MX18 was studied using qRT-PCR (Fig. 7). The basal expression of all three genes in MX18 did not differ from that in WG12. In both strains, expression levels of *Cyp51*, *BcatrD*, and *Bcmfs1* genes significantly increased after treatment with flusilazole at each strain's respective EC_{50} values. Interestingly, the expression of *Cyp51* gene in WG12 was significantly greater than that of the flusilazole-insensitive strain MX18 after treatment with flusilazole ($P < 0.05$), whereas the expression of *BcatrD* in the flusilazole-insensitive strain MX18 was

significantly higher than that in WG12 after treatment with flusilazole ($P < 0.05$). There was no difference in expression of the *Bcmfs1* gene in MX18 compared with the same gene in WG12 after treatment with flusilazole.

Discussion

Gray mold caused by *B. cinerea* has imposed serious limitations on agricultural production all over the world. Due to the lack of highly resistant cultivars in production, control of gray mold relies on fungicide applications. Fungicides with different modes of action, namely, carbendazim, procymidone, and pyrimethanil, have been used in China to control gray mold for several decades. Because of the high genetic variability, short life cycle, and prolific reproduction of *B. cinerea*, repeated use of these fungicides led to multiple-fungicide resistance in field strains of *B. cinerea* worldwide (Myresiotis et al. 2008; Rotolo et al. 2018; Sabrina et al. 2016). Therefore, introduction of new fungicides with different modes of action for consistent control of gray mold is necessary.

The baseline sensitivity of a pathogen to a fungicide is an important reference for assessing resistance risk and monitoring resistance levels. In this research, a total of 95 single-spore baseline strains of *B. cinerea* collected from different regions of Shaanxi Province were tested for their sensitivity to flusilazole. The average EC_{50} value of the 95 strains for inhibiting mycelial growth was $0.093 \mu\text{g/ml}$, which is lower than that of boscalid ($1.07 \mu\text{g/ml}$) and isopyrazam ($0.68 \mu\text{g/ml}$) (Song et al. 2016; Zhang et al. 2007) but higher than that for fluzinam ($0.0201 \mu\text{g/ml}$) (Liu et al. 2019). However, the sensitivity

Fig. 3. Sclerotia production of two *Botrytis cinerea* strains, WG12 and MX18, on PDA (control) or PDA amended with flusilazole (treatment).

Fig. 4. Effect of flusilazole on oxalic acid content between the two *Botrytis cinerea* strains, WG12 and MX18, treated with flusilazole or not. Bars indicate standard deviation of three experiments. Values in columns followed by the same letters are not significantly different according to Fisher's protected LSD test ($P = 0.05$).

Fig. 5. Comparison of pathogenicity between the two *Botrytis cinerea* strains, WG12 and MX18, on strawberry leaves or grapefruit, respectively. Bars indicate standard deviation of three experiments, and n.s. denotes no significant difference according to Fisher's protected LSD test ($P = 0.05$).

Fig. 6. Effect of flusilazole on ergosterol content between the two *Botrytis cinerea* strains, WG12 and MX18, treated with flusilazole or not. Bars indicate standard deviation of three experiments. Asterisk denotes significant difference according to Fisher's protected LSD test ($P = 0.05$).

distribution did not conform to the normal distribution, which suggests that not enough strains were screened and consequently an accurate baseline measurement for sensitivity to flusilazole would require more strains be screened. Nevertheless, the low EC_{50} values and narrow range of frequency distribution indicated that flusilazole has high efficacy in inhibiting the mycelial growth of *B. cinerea*. As expected, no cross-resistance was detected between flusilazole and the other three commonly used fungicides (carbendazim, procymidone, and pyrimethanil). This could be explained by their different modes of action.

On detached strawberry leaves, flusilazole exhibited better control efficacy against both the carbendazim-sensitive and carbendazim-resistant strain of *B. cinerea* compared with carbendazim at the same concentration. Moreover, in greenhouse experiments, flusilazole at 200 $\mu\text{g/ml}$ provided over 85% control efficacy, which was significantly higher than the efficacy of the reference fungicide carbendazim. These data suggest flusilazole has strong antifungal activity and could be used as a potential alternative fungicide in controlling gray mold.

B. cinerea relies on mycelium and sclerotia to survive in diseased plants or soil during winter and summer. Sclerotia commence growth in early spring in temperate regions to produce conidiophores and multinucleate conidia, serving as a primary source of inoculum within a plant (Williamson et al. 2007). *Sclerotinia sclerotiorum* and *B. cinerea* are closely related necrotrophic plant pathogenic fungi (Amsellem et al. 2011). Oxalic acid plays important roles in the pathogenicity and sclerotia development both in *B. cinerea* and *S. sclerotiorum* (Kim et al. 2008; Nakajima and Akutsu 2014). In *S. sclerotiorum*, oxalate-deficient mutants are unable to develop sclerotia (Kim et al. 2008; Williams et al. 2011). In this study, the two *B. cinerea* strains WG12 and MX18 lost the ability to produce sclerotia and oxalic acid content decreased after treatment with flusilazole, indicating that flusilazole could interrupt the life cycle by reducing survival of the pathogen. Moreover, the lower disease severity (smaller lesions) measured when strawberry leaves and grapes were treated with flusilazole suggests decreased pathogenicity of *B. cinerea*. The biocompatibility of resistant strains in the field is an important indicator for assessing the risk of resistance of pathogens to fungicides. Because there were no differences between the two strains WG12 and MX18 in sclerotia production, oxalic acid content, and pathogenicity, in the absence of fungicide treatment, this suggests that *B. cinerea* may exhibit a high risk of resistance to flusilazole.

Ergosterol is the main component of the fungal cell membrane and plays an important role in maintaining the stability of cell membrane structure and function. Ergosterol is not only important for the formation of lipid-raft domains that may regulate the activity of membrane-embedded pumps but also is crucial for plasma membrane stability and adequate selective permeability to avoid the passive diffusion of toxic compounds into the cell (Bagnat et al. 2001; Eisenkolb et al. 2002; Godinho et al. 2018; Parks and Casey 1995; Pasrija et al. 2008). In the current study, ergosterol content decreased significantly after treatment with flusilazole. This was consistent with the mechanism of action of the DMI fungicides that inhibit ergosterol biosynthesis. However, the inhibition rate of ergosterol content in strain MX18, which was the least sensitive to flusilazole of the strains evaluated, was significantly lower than that in the strain most sensitive to flusilazole (WG12), which was consistent with the result of *Neurospora crassa* resistance to DMIs (Zhou and Hollomon 1997).

Similar to many other fungicides, DMIs are also single-site inhibitors, which is reason for the development of pathogen resistance as a result of point mutations in target genes. However, the resistance mechanisms of plant pathogens to DMIs are generally thought to be polygenically governed (Gisi 2014). Previous studies demonstrated that point mutations in the coding region of the sterol 14 α -demethylase gene, protein overexpression, and overexpression of *Cyp51* gene, ABC transporter gene, and MFS transporter gene are all related to the resistance of plant pathogens to DMIs (Canas-Gutierrez et al. 2009; Cools and Fraaije 2013; Leroux and Walker 2011; Schnabel and Jones 2001). For example, point mutations in the *Cyp51* gene had been detected in *U. necator* with high levels of resistance to triadimenol, and the overexpression of ABC

transporter *BcatrD* gene and MFS transporter *Bcmfs1* gene were reported in *B. cinerea* with reduced sensitivity to DMIs (Délye et al. 1997; Hayashi et al. 2001, 2002). It was also demonstrated that ABC transporters are responsible for efflux of drugs from cells of target by directly utilizing the energy generated by ATP hydrolysis. For example, the overexpression of ABC transporter *BcatrD* gene in *B. cinerea* displayed a relatively low accumulation of oxpoconazole (Hayashi et al. 2001). In this study, however, no point mutation in the *Cyp51* gene of MX18 was detected (data not shown), which may be due to the low level of resistance of this strain. As expected, the expression of *Cyp51*, *BcatrD*, and *Bcmfs1* genes both in the most flusilazole-sensitive strain WG12 and the least flusilazole-sensitive strain MX18 were all significantly increased after treatment with flusilazole. Interestingly, the expression of *Cyp51* gene in WG12 was significantly higher than that in MX18 after treatment with flusilazole, whereas the expression of *BcatrD* gene in MX18 was significantly higher than that in WG12 after treatment with flusilazole (Fig. 7). Because the inhibition rate of ergosterol content in WG12 (44.8%) was significantly higher than that in MX18 (23.2%) after treatment with flusilazole, we speculate that when treated with flusilazole, the sensitive strain WG12 overexpressed more *Cyp51* gene than MX18 to synthesize more ergosterol. According to the results from previous studies that ABC transporters are responsible for efflux of drugs from cells (Hayashi et al. 2001, 2002), we speculate that the higher expression level of the *BcatrD* gene in MX18 compared with WG12 accelerated the excretion of flusilazole in vivo, which led to decreased sensitivity of MX18 to flusilazole. Therefore, the lower ergosterol content and the overexpression level of *Cyp51* gene in MX18 suggest that the *Cyp51* gene was critical in ergosterol synthesis and that the *BcatrD* gene plays an important role in drug excretion. These results support the fact that resistance of plant pathogens to DMI fungicides is controlled by multiple genes, such as *Cyp51* and *BcatrD*, and these genes may be important determinants of the resistance of *B. cinerea* to DMI fungicides. The work to induce mutants of *B. cinerea* with a high level of resistance to flusilazole and to determine the accumulation of flusilazole is underway in our laboratory and will provide more valuable information to fully understand the resistance mechanism of plant pathogens to DMIs.

To our knowledge, this study is the first report on the baseline sensitivity of Chinese *B. cinerea* populations to flusilazole and the potential value of this fungicide in controlling gray mold. Although high resistance of *B. cinerea* to DMI fungicides has not yet been detected in China, laboratory-induced DMI-resistant strains have been obtained in many plant pathogens. Strains of *B. cinerea* collected from fields with reduced sensitivity to tebuconazole have been reported (Bolton et al. 2016; Hayashi et al. 2002). Considering that flusilazole is a DMI fungicide, this suggests a high risk of resistance to flusilazole exists in *B. cinerea*. Therefore, to avoid or delay the occurrence of fungicide resistance in controlling gray mold, flusilazole

Fig. 7. Relative expression level of three genes from two strains of *Botrytis cinerea*, WG12 and MX18, that vary in sensitivity to flusilazole (Flu). Values for each gene associated with the same letters are not significantly different according to Fisher's protected LSD test ($P = 0.05$).

should be used interchangeably or mixed with other fungicides with different modes of action.

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