In Vitro Effects of Five Different Classes of Fungicides on Growth and Development of *Botrytis cinerea* Isolated from Tree Peony in China

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Abstract. Gray mold caused by *Botrytis cinerea* has become an important limiting factor for tree peony production. Currently, chemical control is still the main means of managing the disease in China. The objective of this study was to test fungicides with different mechanisms of action in controlling *B. cinerea* on tree peony. The inhibitory efficacy of five fungicides on four asexual stages was measured in the petri dish containing culture medium amended with a tested fungicide at various concentrations. The results showed that carbendazim had the strongest inhibition effect against all four stages of *B. cinerea*, with the EC_{50} values of 0.1037, 0.0563, 0.5578, and 0.0797 $mg \cdot L^{-1}$, respectively. The inhibitory effect of diethofencarb was only slightly less than that of carbendazim on conidia production, germination, and germ tube elongation. The inhibitory effect of procymidone was second only to that of carbendazim on colony expansion. The results indicated that carbendazim and diethofencarb could be used as protective fungicides to spray in the early stage of disease occurrence to inhibit conidia germination and germ tube elongation, so as to reduce the infection rate of *B. cinerea* and prevent disease occurrence. Carbendazim, procymidone, and diethofencarb mainly inhibit the reinfection of *B. cinerea* by inhibiting the growth of mycelium and the production of conidia, so they could be used as control fungicides during the occurrence phase of the disease.

Tree peonies belong to section *Moutan* of the genus *Paeonia* in the family Paeoniaceae. They are an important group of flowering plants referred to as “the king of flowers” as a result of their large flowers, gorgeous color, elegant shape, and rich fragrance. Nowadays, tree peonies have become an internationalized ornamental as well as oil plant and are widely cultivated in many countries (Cheng, 2007; Liu et al., 2018; Zhang et al., 2018). They are unique, traditional, precious plants in China and they have also been cultivated extensively in the United States, France, the Netherlands, and more (Zhao and Tao, 2011). However, gray mold invariably occurs on herbaceous peony plants, especially when grown in the greenhouse (Yang et al., 2017). The symptoms seriously affect the ornamental and commercial values of the plants.

Studies have reported that the main pathogens causing gray mold on tree peony are *Botrytis paeoniae* and *B. cinerea* (Hansen, 2009; Pfleger et al., 1998). Yang et al. (2017) confirmed that *B. cinerea* was the only pathogenic fungus causing tree peony gray mold in Luoyang through the identification of a large number of samples based on morphological characteristics and gene sequencing. It has been recorded that the pathogen can attack more than 200 plant species and results in considerable economic losses worldwide (Williamson et al., 2007). *B. cinerea* can attack different plant organs, including flowers, leaves, fruits, shoots, and soil storage organs. Both conidia and mycelium can serve as a primary inoculum, and they cause disease in many plants. In perennial plants, the dead flowers, leaves, and mummified fruit contain masses of mycelium that can produce conidia and initiate infection (Williamson et al., 2007), which leads to a difficulty in controlling the disease because of the pathogen’s diverse attacking modes and plenty of host plants (Williamson et al., 2007).

In recent years, more plastic greenhouses are widely used in peony-growing areas in China to advance florescence for viewing, especially in parks with peonies. However, this measure also increases the incidence of peony diseases, especially gray mold caused

by *Botrytis cinerea* (Duan, 2009). A disease investigation carried out by Yang et al. (2017) in 2014–15 showed that gray mold is one of the major diseases that affects the normal growth and development of tree peony in Luoyang. This area is one of the main cultivation areas of tree peony; it has a long cultivation history, a large cultivated area, and various varieties in China. Another investigation conducted in 2014 on potted tree peonies grown in a greenhouse showed that gray mold is also the main disease in forcing culture of tree peonies (Yang et al., 2017). In general, the disease has become an important limiting factor of tree peony production and seriously affects the ornamental value of tree peonies both in the open air and in greenhouses.

At present, the effect of using resistant varieties to control the disease is still a long way to go because the genetic relationship among existing tree peony varieties is mostly unclear, which leads to low breeding efficiency and the new variety release rate (Guo et al., 2017; Guo et al., 2018). As a result of the lack of the gray mold-resistant germplasm resources, chemical control is still the main way to reduce the incidence of gray mold on major crops and other plants, although resistance is an inevitable result of evolution. However, there are few reports that document which chemical fungicides are effective in preventing and controlling the gray mold disease at different stages. In our study, five different classes of fungicides—*anilopyrimidine* (e.g., pyrimethanil), *carboxamide* (e.g., boscalid), *benzimidazole* (e.g., carbendazim), *dicarboximide* (e.g., procymidone), and *N-phenylcarbamate* (e.g., diethofencarb)—were used to test the sensitivity of fungicides with different mechanisms of action in controlling the pathogen of gray mold on tree peony in vitro (Avenot and Michailides, 2010; Chapeland et al., 1999; Liu et al., 2016). In addition, the morphological effect of the fungicides on the growth and development of *B. cinerea* was evaluated, and its application methods were analyzed.

Materials and Methods

***B. cinerea* isolate and culture conditions.** The *B. cinerea* isolate (20160401PB) was obtained from tree peony flower buds in a plastic-covered greenhouse in the Sui and Tang dynasties city ruins botanical garden, located in Luoyang, Henan Province, China. The pathogenic fungus was isolated and purified via usual tissue isolation methods (Fang, 1998) and then the isolate was cultured on a potato dextrose agar (PDA) plate medium to observe colony morphology (Tian et al., 2019). After sporulation, the morphological characteristics were observed, photomicrographs of conidiophores and conidia were obtained, and conidia size was measured.

The fungus was grown in petri dishes containing PDA medium and were kept in an incubator at 25 °C. To prepare the inoculum, sterile distilled water was added to

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the petri dishes to cover the colony, and the conidia were collected with the aid of a glass rod. For all assays, the inoculum concentration was adjusted to 10^5 conidia/mL after counting in a hemocytometer.

Fungicides. The fungicides used for in vitro bioassays were technical grade and were the following: boscalid (96.2%; BASF SE, Ludwigshafen, Germany), carbendazim (97.0%; Zhenjiang Chemical Factory, Zhenjiang, China), diethofencarb (97.4%, Zhenjiang Chemical Factory), procymidone (98.0%; Sumitomo Chemical Co. Ltd., Kyoto, Japan), and pyrimethanil (95.2%; Fengdeng Chemical Factory, Changzhou, China). Stock solution ($10 \text{ mg}\cdot\text{mL}^{-1}$) diethofencarb was prepared by dissolving in methanol (99.5%). Boscalid was dissolved in acetone to obtain $10\text{-mg}\cdot\text{mL}^{-1}$ stock solutions. Carbendazim was dissolved in 0.1 M HCl to obtain a $10\text{-mg}\cdot\text{mL}^{-1}$ stock solution. Procymidone and pyrimethanil were dissolved in methanol to obtain $10\text{-mg}\cdot\text{mL}^{-1}$ stock solutions (Liu et al., 2016). All stock solutions were stored at 4°C in the dark to preserve fungicidal toxicity. The correct volume was added to the PDA medium to generate the final concentrations of each fungicide. A volume of 15 mL fungicide-amended PDA media was then poured immediately into sterilized petri dishes (diameter, 90 mm). The controls were prepared without fungicide by adding the same volume of HCl or methanol. It was reported previously that the volume of HCl and methanol comprised less than 0.25% of each stock solution and the concentration did not affect mycelial growth of *B. cinerea* (Liu et al., 2016).

The range of fungicide concentrations for the assays were defined in preliminary experiments in medium and were the following: diethofencarb, 0.3125, 0.625, 1.25, 2.5, and $5.0 \text{ mg}\cdot\text{L}^{-1}$; boscalid, 0.625, 1.25, 2.5, 5.0, and $10.0 \text{ mg}\cdot\text{L}^{-1}$; procymidone, 2.5, 5.0, 10.0, 20.0, and $40.0 \text{ mg}\cdot\text{L}^{-1}$; carbendazim, 0.0390625, 0.078125, 0.15625, 0.3125, and $0.625 \text{ mg}\cdot\text{L}^{-1}$; and pyrimethanil, 3.75, 7.50, 15.0, 30.0, and $60 \text{ mg}\cdot\text{L}^{-1}$ (Avenot and Michailides., 2010; Liu et al., 2016).

Sensitivity to boscalid, carbendazim, diethofencarb, and procymidone was tested in fungicide-amended PDA. Pyrimethanil sensitivity was tested on a synthetic medium as described by Yourman and Jeffers (1999).

Effect of the tested fungicides on mycelium growth rate. Mycelial plugs (diameter, 5 mm) were cut from the growing edge of a 5-d-old colony and then placed on the PDA plates amended with different concentrations of fungicides and incubated at 25°C in the dark. Colony growth rate (measured in millimeters per 5 d) was determined on PDA medium at different concentrations. Three plates were evaluated for each fungicide and an average colony diameter was calculated.

Mean colony diameter (minus the diameter of the plug) was measured for each treatment and expressed as a percentage of growth inhibition. The median effective con-

centration (EC_{50}) of five fungicides for *B. cinerea* isolate was calculated by linear regression of the relative percentage of growth inhibition against the log-transformed fungicide concentration.

Effect of tested fungicides on conidia production. To assess the effect of tested fungicides on conidia production of *B. cinerea*, 5-mm mycelial plugs from the actively growing 5-d-old colonies were placed onto PDA plates containing a series of different concentrations of each fungicide. After incubation at 25°C in the dark for 14 d, conidia were collected from plates by pouring 5 mL sterile water containing 0.2% Tween-80 onto each plate and filtering the suspension through four layers of lens tissue. The conidia were counted with a hemocytometer using a microscope. Three plates for each concentration were washed to calculate the amount of conidia, and the experiment was performed twice.

Effect of tested fungicides on germination of conidia. A conidia suspension was prepared to evaluate the effect of fungicides on conidia germination. Mycelial plugs (diameter, 5 mm) were cut from the edge of a 5-d-old colony and placed on PDA plates. After incubation for 14 d at 25°C , conidia were collected from the plates by pouring 5 mL sterile water containing 0.2% Tween-80 onto each plate and filtering the suspension through four layers of sterile gauze. The filtrate was then centrifuged at $4000 g_n$ for 10 min. The supernatant was discarded and the conidia were suspended in sterile water to a concentration of 10^5 conidia/mL. One hundred microliters of suspension was added to a water agar (WA) plate, each with one of series of different concentrations of fungicides. After incubation at 25°C in the dark for 2, 4, 6, and 8 h continuously, the length of the germ tube was observed using a light microscope at $20\times$ magnification. The criterion for germination is that the length of the germ tube is more than half the maximum diameter of the spore. When the conidia germination rate of the WA plate without fungicide reached more than 90%, the number of conidia in the treatment group was counted with a hemocytometer using a microscope (Liu et al., 2016).

Effect of the tested fungicides on germ tube elongation. The conidia suspension (10^5 conidia/mL) was prepared according to the method described earlier. One hundred microliters of suspension containing about 10^5 conidia was added to WA plates, each of which was amended with a series of different concentrations of each fungicide. After incubation at 25°C in the dark for 6 and 8 h, the length of the germ tube was measured using a light microscope at $20\times$ magnification. The morphology of the conidia and the germ was

were observed after measurement. Approximately 200 conidia were counted to calculate the germination rate and germ tube length. A statistical analysis was performed on triplicates of 200 conidia. The experiment was performed twice.

Data analysis. The EC_{50} value for each fungicide was estimated by linear regression of the probit-transformed relative inhibition value on $\log_{10}$ -transformed fungicide concentration. Results represent regressions performed on three replications per fungicide. The frequency distribution of EC_{50} values for each fungicide was tested for normality using the Shapiro-Wilk test (PROC UNIVARIATE) for normality using SAS (version 9.4; SAS Institute, Cary, NC). Data from each fungicide repeat were analyzed separately, and the EC_{50} values obtained for each fungicide in Expts. 1 and 2 were compared using a paired *t* test ($\alpha = 0.05$). The combined mean EC_{50} values were compared by analysis of variance to determine differences among fungicides.

Results

In vitro inhibitory effects of tested fungicides on mycelium growth rate. All of the five fungicides used in the sensitivity tests in vitro inhibited the growth of *B. cinerea* isolate at different levels (Table 1). Comparatively, carbendazim had the greatest inhibitory effects, with an average EC_{50} value of $0.1037 \text{ mg}\cdot\text{L}^{-1}$. For the other four fungicides, the mean EC_{50} values ranged from 3.8525 to $35.5201 \text{ mg}\cdot\text{L}^{-1}$ (Table 1). In addition, the inhibitory effects showed an obvious dose-dependent manner; when the concentration of fungicide increased, the colony diameter decreased (Fig. 1).

Effect of tested fungicides on conidia production. Our study found that the *B. cinerea* isolate began to produce massive amounts of conidia after 10 d of continuous culture on nonfungicide-amended PDA plates. However, the amount of conidia produced on fungicide-amended PDA plates correlated negatively with the concentration of the fungicides. Different fungicides have different inhibition rates at different concentrations. The results show that conidia production decreased gradually with an increase in the concentration of the tested fungicides. In general, carbendazim had the strongest inhibitory effect on conidia production of *B. cinerea*, followed by diethofencarb and boscalid, with mean EC_{50} values of 0.0563, 2.5755, and $3.8036 \text{ mg}\cdot\text{L}^{-1}$, respectively (Table 2). The inhibition effects of procymidone and pyrimethanil were relatively weak, with mean EC_{50} values of 6.4341 and $7.7371 \text{ mg}\cdot\text{L}^{-1}$, respectively (Table 2).

Table 1. Inhibitory effect of five fungicides on mycelium growth of *B. cinerea* isolated from tree peony.

Fungicide	Toxicity regression equation	EC_{50} ($\text{mg}\cdot\text{L}^{-1}$)	Correlation coefficient
Carbendazim	$y = 10.1148 + 5.1969x$	0.1037	0.9569
Procymidone	$y = 3.8663 + 1.9354x$	3.8525	0.9909
Diethofencarb	$y = 4.0588 + 0.9288x$	10.3122	0.9801
Boscalid	$y = 3.9154 + 0.8643x$	17.9860	0.9699
Pyrimethanil	$y = 1.9317 + 1.9790x$	35.5201	0.9804

Fig. 1. Inhibitory rates of five fungicides on mycelium growth of *B. cinerea* on tree peony. CK, control group.

Effect of tested fungicides on conidia germination. Results of a preliminary experiment indicated that the germination rate of conidia of *B. cinerea* could reach more than 95% after 8 h of inoculation of conidia suspension on nonfungicide-amended PDA plate. The results also show that all the tested fungicides had a certain inhibitory effect on conidia germination of *B. cinerea* on fungicide-amended PDA medium, and the inhibition rate increased with an increase in the concentration of fungicides (Table 3). Carbendazim had the strongest inhibitory rate on conidia germination, followed by diethofencarb and pyrimethanil, with mean EC_{50} values of 0.5578, 1.9264, and 7.1306 $mg \cdot L^{-1}$, respectively (Table 3). Boscalid and procymidone were relatively weak, with mean EC_{50} values of 15.9119 and 16.8471 $mg \cdot L^{-1}$, respectively.

Effect of tested fungicides on germ tube elongation. The preliminary experiment indicated that the common germination rate of conidia of *B. cinerea* could reach a length of 53 μm after 10 h of inoculation of conidia suspension on nonfungicide-amended PDA medium. The results show that the germ tube elongation of *B. cinerea* was inhibited in the presence of fungicides, and the inhibitory effects correlated positively with the concentration of fungicides (Table 4). As shown in Fig. 2, carbendazim had the strongest inhibitory effects on germ tube elongation, followed by diethofencarb, with mean EC_{50} values of 0.0797 and 1.0221 $mg \cdot L^{-1}$, respectively (Table 4). The inhibition effects of boscalid and pyrimethanil were relatively weak, with mean EC_{50} values of 3.7089 and 4.3374 $mg \cdot L^{-1}$, respectively. Procymidone had the lowest inhibitory effects on germ tube elongation of *B. cinerea*.

Table 2. Effects of five fungicides on conidia production of *B. cinerea* isolated from tree peony.

Fungicide	Toxicity regression equation	EC_{50} ($mg \cdot L^{-1}$)	Correlation coefficient
Carbendazim	$y = 9.6095 + 3.6900x$	0.0563	0.9761
Diethofencarb	$y = 4.6142 + 0.9390x$	2.5755	0.9872
Boscalid	$y = 4.4546 + 0.9401x$	3.8036	0.9881
Procymidone	$y = 3.4128 + 1.9632x$	6.4341	0.9738
Pyrimethanil	$y = 3.2377 + 1.9833x$	7.7371	0.9713

Table 3. Effects of five fungicides on conidia germination of *B. cinerea* isolated from tree peony.

Fungicide	Toxicity regression equation	EC_{50} ($mg \cdot L^{-1}$)	Correlation coefficient
Carbendazim	$y = 5.1762 + 0.6948x$	0.5578	0.9913
Diethofencarb	$y = 4.8149 + 0.6499x$	1.9264	0.9681
Pyrimethanil	$y = 3.8912 + 1.2997x$	7.1306	0.9687
Boscalid	$y = 4.1720 + 0.6890x$	15.9119	0.9934
Procymidone	$y = 3.6360 + 1.1121x$	16.8471	0.9803

Table 4. Effects of five fungicides on germ tube elongation of *B. cinerea* isolated from tree peony.

Fungicide	Toxicity regression equation	EC_{50} ($mg \cdot L^{-1}$)	Correlation coefficient
Carbendazim	$y = 6.1947 + 1.0876x$	0.0797	0.9527
Diethofencarb	$y = 4.9917 + 0.8708x$	1.0221	0.9935
Boscalid	$y = 4.4399 + 0.9840x$	3.7089	0.9553
Pyrimethanil	$y = 4.3542 + 1.0135x$	4.3374	0.9796
Procymidone	$y = 3.2753 + 1.8175x$	8.8908	0.9972

In addition, our results show that germ tube length of *B. cinerea* became significantly shorter after treatment with four fungicides (Carbendazim, Procymidone, Boscalid, and Pyrimethanil). The germ tubes of *B. cinerea* were even enlarged at their base after treatment with procymidone and boscalid.

Discussion

In this study, five different classes of fungicides were measured for their sensitivity on the growth and development of *B. cinerea*

isolated from tree peony. The results indicate that carbendazim had the greatest inhibitory effect among the tested fungicides on colony expansion, conidia formation, germ tube germination, and elongation of *B. cinerea*, with inhibitory effects correlating positively to concentration. The inhibitory effect of diethofencarb was only slightly less than that of carbendazim on conidia production, germination, and germ tube elongation, with average EC_{50} values of 2.5755, 1.9264, and 1.0221 $mg \cdot L^{-1}$, respectively. The inhibitory effect of procymidone was second only to that of

Fig. 2. Effects of five fungicides on germ tube elongation and malformation of *B. cinerea* on tree peony. (A) Carbendazim. (B) Procymidone. (C) Diethofencarb. (D) Boscalid. (E) Pyrimethanil. CK, control group. The lowercase letters indicate the concentration of the corresponding fungicide. Bars = 20 µm.

carbendazim on colony expansion. In contrast, pyrimethanil had a relatively weak inhibitory effect on colony expansion and conidia production of *B. cinerea*; procymidone had the least effect on conidia germination and germ tube elongation.

It has been demonstrated that mycelium and conidia play an important role in the occurrence and epidemic of gray mold disease as the primary infection sources (Williamson et al., 2007). The results of our study indicate that all of the five tested fungicides had different degrees of inhibitory effects on *B. cinerea*, which causes gray mold on tree peony. Although they cannot kill the conidia directly, they can reduce the germination rate of conidia and deform the germ tube, which had a strong inhibitory effect on the elongation of the germ tube. As a result, the fungicides reduced the ability of *B. cinerea* to infect and produce conidia on the host. Therefore, these fungicides are expected to be effective for chemical control of gray mold on peony.

However, different application methods should be adopted according to their different morphological and toxicological effects. Carbendazim and diethofencarb could be used as protective fungicides to apply during the early stage of disease occurrence to inhibit conidia germination and germ tube elongation, and thus reduce the infection rate of *B. cinerea* and prevent disease occurrence. Carbendazim, procymidone, and diethofencarb mainly inhibit the reinfection of *B. cinerea* by

inhibiting the growth of mycelium and the production of conidia, so they could be used as a control fungicide during the occurrence phase of the disease.

In conclusion, fungicide resistance in *B. cinerea* has become an important problem in vegetables, fruit trees, and flowering plants (Rodríguez et al., 2014). In recent years, the fungicide resistance of boscalid, carbendazim, pyrimethanil, and diethofencarb has been reported frequently in *B. cinerea* field isolates on vegetables and fruit trees (Fernández-Ortuño et al., 2016a, b; Rodríguez et al., 2014). Tree peony growers should appropriately use protectant and control fungicides to delay the emergence of fungicide resistance in strains of gray mold infecting peony.

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