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·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 reinfec-
tion 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 *Moudan* of the genus *Paeonia* in the family *Paeoniaeaceae*. 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 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 paeonae* 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 be inoculum, and they cause disease in many plants. In perennial plants, the dead flowers, leaves, and mmurified 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—anilinopyrimidine (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...
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 mg·mL^−1) diethofencarb was prepared by dissolving in methanol (99.5%). Boscalid was dissolved in acetone to obtain 10-mg·mL^−1 stock solution. Procymidone and pyrimethanil were dissolved in methanol to obtain 10-mg·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 mg·L^−1; boscalid, 0.625, 1.25, 2.5, 5.0, and 10.0 mg·L^−1; procymidone, 2.5, 5.0, 10.0, 20.0, and 40.0 mg·L^−1; carbendazim, 0.0390625, 0.078125, 0.15625, 0.3125, and 0.625 mg·L^−1; and pyrimethanil, 3.75, 7.50, 15.0, 30.0, and 60 mg·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 in fungicide-amended PDA. Pyrimethanil was tested at different levels (Table 1). Comparatively, carbendazim had the greatest inhibitory effects, with an average EC_50 value of 0.1037 mg·L^−1. For the other four fungicides, the mean EC_50 values ranged from 3.8525 to 35.5201 mg·L^−1 (Table 1).

**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 (*α* = 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 strongest inhibitory effects, with an average EC_50 value of 1985 mg·L^−1. For the other four fungicides, the mean EC_50 values ranged from 3.8525 to 35.5201 mg·L^−1 (Table 1).

**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 lens tissue. The conidia were counted with a hemocytometer using a microscope. Three plates for each concentration were washed to calculate the amplitude 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 lens tissue. The filtrate was then centrifuged at 4000 g 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× 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× magnification. The morphology of the conidia and the germ was observed after measurement. Appropriately 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.

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

| Fungicide   | Toxicity regression equation | EC_50 (mg·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                 |
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 EC50 values of 0.5578, 1.9264, and 7.1306 mg L−1, respectively (Table 3). Boscalid and procymidone were relatively weak, with mean EC50 values of 15.9119 and 16.8471 mg 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 EC50 values of 0.0797 and 1.0221 mg L−1, respectively (Table 4). Boscalid and pyrimethanil were relatively weak, with mean EC50 values of 3.7089 and 4.3374 mg L−1, respectively. Procymidone had the lowest inhibitory effects on germ tube elongation of *B. cinerea*.

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 EC50 values of 2.5755, 1.9264, and 1.0221 mg L−1, respectively. The inhibitory effect of procymidone was second only to that of...
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 (Rodriguez 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; Rodriguez 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.

 Literature Cited
Avenot, H.F. and T.J. Michailides. 2010. Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. Crop Prot. 29:643–651.
Chapeland, F., R. Fritz, C. Lanen, M. Gredt, and P. Leroux. 1999. Inheritance and mechanisms of resistance to anilinopyrimidine fungicides in Botrytis cinerea (Botryotinia fuckeliana). Pest. Biochem. Physiol. 64:85–100.
Cheng, F.Y. 2007. Advances in the breeding of tree peonies and a cultivar system for the cultivar group. Intl. J. Plant Breed. 1:89–104.
Duan, Y.B. 2009. Study on the identification and biological characteristics of pathogenic fungi of peony leaf spot. Master thesis. Henan University of Science and Technology, Luoyang, China (in Chinese).
Fang, Z.D. 1998. Research methods of plant disease. 3rd ed. China Agriculture Press, Beijing (in Chinese).
Fernández-Ortuño, D., J.A. Tores, M. Chamorro, A. Pérez-Garcia, and A. de Vicente. 2016a. Characterization of resistance to six chemical classes of site-specific fungicides registered for gray mold control on strawberry in Spain. Plant Dis. 100:2234–2239.
Fernández-Ortuño, D., J.A. Tores, A. Pérez-Garcia, and A. de Vicente. 2016b. First report of fludioxonil resistance in Botrytis cinerea, the causal agent of gray mold, from strawberry fields in Spain. Plant Dis. 100:1779.
Guo, Q., L.L. Guo, L. Zhang, L.X. Zhang, H.L. Ma, D.L. Guo, and X.G. Hou. 2017. Construction of a genetic linkage map in tree peony (Paeonia Sect. Moutan) using simple sequence repeat (SSR) markers. Scientia Hort. 219:294–301.
Guo, L.L., D.L. Guo, W. Zhao, and X.G. Hou. 2018. Newly developed SSR markers reveal genetic diversity and geographical clustering in Paeonia suffruticosa based on flower colour. J. Hort. Sci. Biotechnol. 93:416–424.
Hansen, M.A. 2009. Botrytis blight of peony. Virginia Cooperative Extension, Petersburg, VA.
Liu, S.M., Z.P. Che, and G.Q. Chen. 2016. Multiple-fungicide resistance to carbendazim, diethofencarb, procymidone, and pyrimethanil in field isolates of Botrytis cinerea from tomato in Henan Province, China. Crop Prot. 84:56–61.
Liu, P., Y. Zhang, Y.F. Xu, X.Y. Zhu, X.F. Xu, S. Chang, and R.X. Deng. 2018. Three new monoterpene glycosides from oil pea seed cake. Ind. Crops Prod. 111:371–378.

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.
Pfleger, F.L., J.L. Fetzer, and W.J. White-McDougall. 1998. Diseases of peony. Regents of the University of Minnesota. <http://purl.umn.edu/50166>.

Rodríguez, A., A. Acosta, and C. Rodríguez. 2014. Fungicide resistance of *Botrytis cinerea* in tomato greenhouse in the Canary Islands and effectiveness of non-chemical treatments against gray mold. World J. Microb. Biot. 30:2397–2406.

Tian, Y.E., Z.P. Che, D. Sun, Y.Y. Yang, X.M. Lin, S.M. Liu, X.Y. Liu, and J. Gao. 2019. Resistance identification of tree peony cultivars of different flowering time to gray mold pathogen *Botrytis cinerea*. HortScience 54:328–330.

Williamson, B., B. Tudzynski, P. Tudzynski, and J.A.L. Van Kan. 2007. *Botrytis cinerea*: The cause of gray mold disease. Mol. Plant Pathol. 8:561–580.

Yang, R.X., P. Liu, Z.H. Wang, and G.D. Song. 2017. Identification of gray mold pathogen on tree peony *Paeonia suffruticosa* in Luoyang. J. Plant Prot. 44:623–629 (in Chinese).

Yourman, L.F. and S.N. Jeffers. 1999. Resistance to benzimidazole and dicarboximide fungicides in greenhouse isolates of *Botrytis cinerea*. Plant Dis. 83:569–575.

Zhang, Y., P. Liu, J.Y. Gao, X.S. Wang, M. Yan, N.C. Xue, C.X. Qu, and R.X. Deng. 2018. *Paeonia veitchii* seeds as a promising high potential by-product: Proximate composition, phytochemical components, bioactivity evaluation and potential applications. Ind. Crops Prod. 125:248–260.

Zhao, D.Q. and J. Tao. 2011. Research progress of herbaceous peony cut flowers. Jiangsu Agr. Sci. 39:286–289 (in Chinese).