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Effectiveness of Different Classes of Fungicides on *Botrytis cinerea* Causing Gray Mold on Fruit and Vegetables

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***Botrytis cinerea* is a necrotrophic pathogen causing a major problem in the export and post-harvest of strawberries. Inappropriate use of fungicides leads to resistance among fungal pathogens. Therefore, it is necessary to evaluate the sensitivity of *B. cinerea* to various classes of fungicide and to determine the effectiveness of different concentrations of commonly used fungicides. We thus evaluated the effectiveness of six classes of fungicide in inhibiting the growth and development of this pathogen, namely, fludioxonil, iprodione, pyrimethanil, tebuconazole, fenpyrazamine, and boscalid. Fludioxonil was the most effective (EC_{50} < 0.1 μg/ml), and pyrimethanil was the least effective (EC_{50} = 50 μg/ml), at inhibiting the mycelial growth of *B. cinerea*. Fenpyrazamine and pyrimethanil showed relatively low effectiveness in inhibiting the germination and conidial production of *B. cinerea*. Our results are useful for the management of *B. cinerea* and as a basis for monitoring the sensitivity of *B. cinerea* strains to fungicides.**

***Keywords* : *Botrytis cinerea*, fungicide, strawberry**

Strawberry is an important commercial fruit crop that is grown worldwide due to its attractive taste (Jang et al., 2011). South Korea is the fifth largest producer of strawberries, producing approximately 230,000 tons in 2012, behind the United States, Spain, Turkey, and Egypt (Na et al., 2013). However, many strawberry diseases have become major hindrances to strawberry production and export, including anthracnose crown rot caused by *Colletotrichum fructicola*, gray mold caused by *Botrytis cinerea* (teleomorph: *Botryotinia fuckeliana*), Fusarium wilt caused by *Fusarium oxysporum* f. sp. *fragariae*, and powdery mildew caused by *Podosphaera aphanis* (Je et al., 2015; Nam et al., 2005, 2014; Williamson et al., 2007). Among these diseases, gray mold is the most common and severe pre- and post-harvest disease; the gray mold infection rate of a strawberry cultivar, Seolhyang, which represents approximately 50% of strawberry production in South Korea, is greater than 50% in this country (Lee and Chae, 2012). *B. cinerea* gains entry to strawberry tissues at an early stage of crop development and remains quiescent there until the fruit starts to ripen (Williamson et al., 2007). After the strawberry fruit has been transported to market, fungal growth resumes, becomes serious, and rapidly spreads to adjacent strawberries. This implies that management of the fungal disease is required when strawberries grow and after they have been harvested.

Different classes of fungicide have been applied to manage gray mold, such as fludioxonil, tebuconazole, iprodione, and boscalid. However, owing to the improper application of fungicides and the high genetic variability of *B. cinerea*, strains resistant to fungicides have emerged and become threats to producers. For example, benzimidazole fungicides, which act by binding to fungal microtubules and inhibiting hyphal growth, were highly effective against gray mold in the late 1960s, but strains resistant to these fungicides started to emerge within a few years of their application, and the fungicides are less effective

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(Beever and Brien, 1983; Shim et al., 2014). In addition, strains resistant to aniline-pyrimidine, dicarboximide, hydroxyaniline, and phenylpyrrole have been reported (Fernández-Ortuño et al., 2012). Thus, it is necessary to apply appropriate fungicides and determine the efficacy of currently used ones. Here, as an initial assessment, we evaluated the effect of six classes of fungicide on *B. cinerea* development.

We isolated *B. cinerea* from strawberry fruit with symptoms of gray mold in a greenhouse in Chuncheon, Gangwon Province, in December 2014. Strawberries showing gray mold symptoms were scraped using a toothpick, inoculated on potato dextrose agar (PDA; MB Cell, Seoul, Korea), and incubated at 25°C in the light for 2 days. The isolate was sub-cultured from hyphal tips to separate it from contaminating bacteria, grown for 2 days, and subjected to single spore isolation. Stock of the isolate was stored at –75°C in 50% glycerol. The identity of the *B. cinerea* isolate was confirmed based on a sequence analysis of the internal transcribed spacer (ITS) region and morphological characteristics. Briefly, *B. cinerea* was cultured on PDA for 3 days and genomic DNA was extracted from the 10- to 20-mg fungal mass using the drilling method described by Chi et al. (2009). The quantity and quality of the extracted DNA were assessed using a BioSpectrometer (Eppendorf, Hamburg, Germany). The ITS region was amplified with the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) in a 20-µl reaction mixture using *Pfu* polymerase (Gálvez et al., 2016) (Elpis, Daejeon, Korea). DNA sequencing was performed by Macrogen Inc. (Daejeon, Korea). BLAST searches of the obtained sequences were performed in the National Center for Biotechnology Information (NCBI) database. An approximately 600-bp sequence of the ITS region was subjected to a BLAST search in GenBank, the NCBI database, which revealed 99.9% identity to *B. cinerea* at the nucleotide level.

Table 1. Fungicides used in this study

Chemical group	Active ingredient	Concentration (%)	Formulation
Phenylpyrrole	Fludioxonil	20.0	SC
Triazole	Tebuconazole	50.0	SC
Dicarboximide	Iprodione	20.0	WP
Anilino-pyrimidine	Pyrimethanil	37.0	SC
Anilide	Boscalid	49.3	WG
Pyrazole	Fenpyrazamine	30.0	SC

SC, suspension concentrate; WP, wettable powder; WG, water-dispersible granule.

To evaluate the inhibitory effect of the six fungicides on the mycelial growth of *B. cinerea* (Table 1), these fungicides were purchased and analyzed to determine the concentrations that caused 50% and 90% reduction in mycelial growth compared with that in their absence. Different concentrations of each fungicide were added to PDA plates, which were then inoculated with mycelial agar plugs (5 mm in diameter) from 3-day-old PDA culture plates. The linear growth of the mycelia was measured after 5 days of inoculation at 25°C in the dark. The growth inhibition rate was determined by comparing the linear growth on PDA supplemented with fungicides to that on PDA without a fungicide.

The phenylpyrrole fungicide fludioxonil was the most effective, inhibiting 93.7% and 100% of mycelial growth at concentrations of 0.1 and 1 µg/ml, respectively (Fig. 1). The inhibitory effect of the dicarboximide fungicide iprodione was greatly increased at a concentration of 1 µg/ml, compared with its effect at 0.1 µg/ml. At a concentration of 10 µg/ml, iprodione and tebuconazole achieved almost complete inhibition of the mycelial growth of *B. cinerea*. The inhibitory effect of boscalid increased only to a concentration of 10 µg/ml, but no increases in its effects were observed even at higher concentrations. The inhibitory rates of fenpyrazamine on mycelial growth ranged from 51.3% to 60.8% at different concentrations, indicating that its inhibitory effect was not highly affected by its concentration. The maximum inhibitory effect of pyrimethanil was 85% at 3,000 µg/ml. Based on these results, 50% effective concentration (EC_{50}) and EC_{90} values were estimated (Gang et al., 2015; Matheron and Porchas, 2000). Fludioxonil showed the lowest EC_{50} value of < 0.1 µg/ml, and pyrimethanil showed the highest of 50 µg/ml (Table 2). Meanwhile, tebuconazole, iprodione, boscalid,

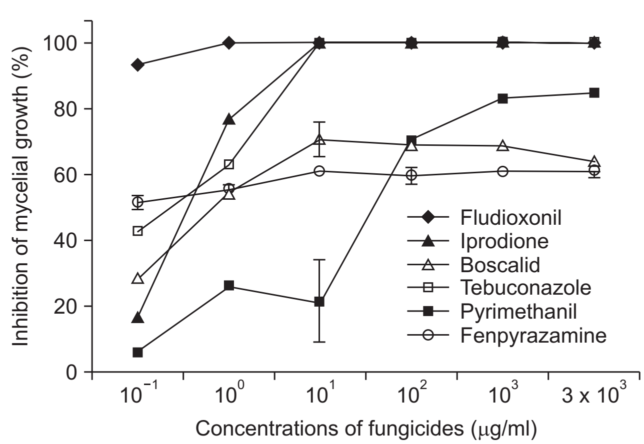

Fig. 1. Effects of fludioxonil, tebuconazole, iprodione, pyrimethanil, boscalid, and fenpyrazamine on the mycelial growth inhibition of *Botrytis cinerea*.

and fenpyrazamine showed EC_{50} values in the range of 0.3 to 0.9 μg/ml. The EC_{90} value of fludioxonil was still < 0.1 μg/ml, while those of tebuconazole and iprodione were 5 and 3 μg/ml, respectively. The EC_{90} values of pyrimethanil, boscalid, and fenpyrazamine were > 3,000 μg/ml.

To evaluate the inhibitory effect of six fungicides on the germination of *B. cinerea*, conidia of *B. cinerea* were collected by rubbing the surface of a 5-day-old PDA plate with sterile distilled water (SDW). The conidial suspension was diluted to approximately 3×10^3 conidia/ml in SDW. The method of conidial filtering, washing, and counting was described by Shin et al. (2014). The diluted conidial suspension was mixed with 0.1, 1, 10, 100, 1,000, and 3,000 μg a.i./ml of each fungicide and placed on slide glasses covered with cover glasses. After 24 h at 25°C in the dark, the conidial germination rate was measured using a minimum of 100 conidia per replicate.

In the inhibition of *B. cinerea* germination (Fig. 2), fludioxonil was the most effective fungicide at a concentration of 0.1 μg/ml, inhibiting up to 91% of the germination of *B. cinerea*; the other five fungicides were less effective, inhibiting germination at a rate of less than 50%.

Table 2. EC_{50} and EC_{90} values of the mycelial growth of *Botrytis cinerea* for six fungicides

Active ingredient	EC_{50} (μg/ml)	EC_{90} (μg/ml)
Fludioxonil	< 0.10	< 0.10
Tebuconazole	0.30	5.00
Iprodione	0.40	3.00
Pyrimethanil	50.00	> 3,000
Boscalid	0.70	> 3,000
Fenpyrazamine	0.90	> 3,000

At a concentration of 10 μg/ml, the inhibitory effects of tebuconazole, iprodione, and boscalid were significantly increased (80%, 96%, and 99% inhibition), whereas those of fenpyrazamine and pyrimethanil were still low, inhibiting germination at 23% and 7%, respectively. However, the inhibitory effects of fenpyrazamine and pyrimethanil were greatly increased at a concentration of 100 μg/ml. At a concentration of 3,000 μg/ml, all fungicides reached 100% inhibition of the germination of *B. cinerea*.

To measure the inhibitory effects of the six fungicides on the conidiation of *B. cinerea*, an isolate of this species was cultured on PDA for 5 days at 25°C in the light. Conidia were collected by rubbing the surface of the PDA with 5 ml of SDW and counted with a hemocytometer. The inhibition rate of conidiation was measured by comparing the number of conidia on PDA supplemented with fungicides to those on PDA with no fungicide. All experiments including those on the inhibitory effects on growth, germination, and conidiation were conducted in triplicate and repeated three times. All data were processed with the SigmaStat statistical software package (SPSS Science, Chicago, IL, USA). Error bars represent 95% confidence intervals and means with non-overlapping 95% confidence intervals were considered significantly different (Glantz, 1992).

For growth and germination inhibition, fludioxonil was the most effective fungicide at 0.1 μg/ml, inhibiting approximately 100% of the conidiation of *B. cinerea* (Fig. 3). Boscalid was also highly effective (91% inhibition) at a concentration of 0.1 μg/ml, but the remaining fungicides—tebuconazole, iprodione, pyrimethanil, and fenpyrazamine—were less effective (62%, 55%, 37%, and 35% inhibition, respectively). The inhibitory effect of tebuconazole was greatly increased at a concentration

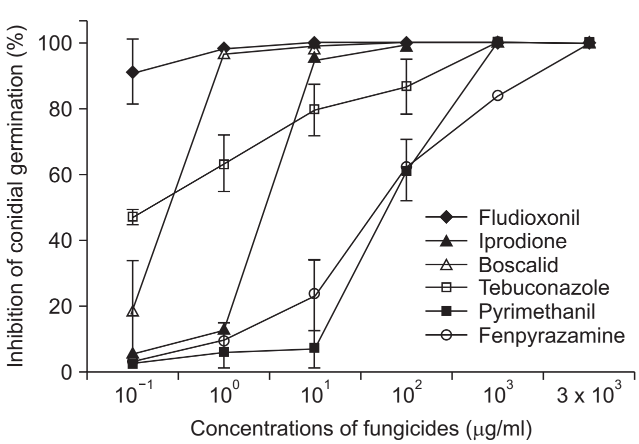

Fig. 2. Effects of fludioxonil, tebuconazole, iprodione, pyrimethanil, boscalid, and fenpyrazamine on the inhibition of germination by *Botrytis cinerea*.

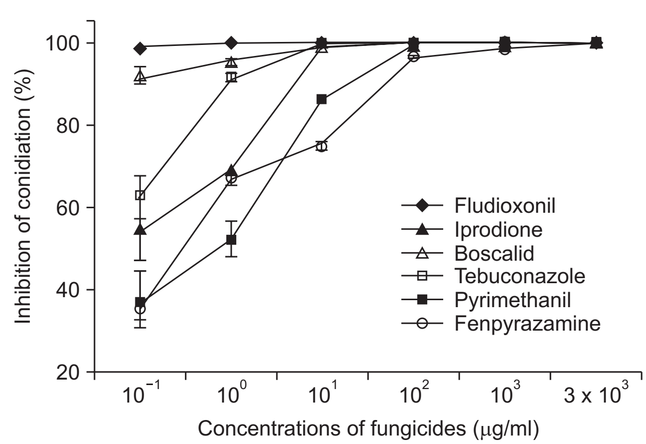

Fig. 3. Effects of fludioxonil, tebuconazole, iprodione, pyrimethanil, boscalid, and fenpyrazamine on the inhibition of conidiation by *Botrytis cinerea*.

of 1 μg/ml (91%), whereas iprodione, pyrimethanil, and fenpyrazamine were less effective. At a concentration of 100 μg/ml, all fungicides exceeded 95% inhibition, indicating that the inhibitory effect is highly affected by their concentration.

Collectively, the phenylpyrrole fungicide fludioxonil was the most effective fungicide in the inhibiting the mycelial growth, germination, and conidiation of *B. cinerea*. Several field or *in vitro* studies have shown that fludioxonil is one of the most effective fungicides for controlling *B. cinerea* by interfering with the osmotic signal transduction pathway of fungi (Kim et al., 2007; Ochiai et al., 2002; Petit et al., 2011). For example, Myresiotis et al. (2007) calculated EC_{50} values of 0.001 to 0.008 μg/ml fludioxonil in the inhibition of mycelial growth of *B. cinerea* isolated from tomato, cucumber, and eggplant stems, leaves, flowers, or fruit. Sholberg et al. (2005) calculated an EC_{50} value of 0.04 μg/ml in the mycelial growth inhibition of *B. cinerea* isolated from stored apples. These results are consistent with our data. The EC_{50} and EC_{90} values of the aniline-pyrimidine fungicide pyrimethanil, which affects the methionine biosynthesis of fungi, were highest among the fungicides used for the inhibition of *B. cinerea* (50 and 3,000 μg/ml, respectively). Supportively, Myresiotis et al. (2007) also reported EC_{50} values of 0.03 to 75 μg/ml for pyrimethanil in the mycelial growth of *B. cinerea*. Taken together, our result indicates that *B. cinerea* is less sensitive to pyrimethanil than the other fungicides. In the mycelial growth inhibition of *B. cinerea*, Kim and Xiao (2010) estimated EC_{50} values of 0.01 to 69.91 μg/ml for boscalid, Myresiotis et al. (2007) estimated EC_{50} values of 0.1 to 1.42 μg/ml for iprodione, and Köycü et al. (2012) estimated EC_{50} values of 0.03 to 1 μg/ml for tebuconazole. These results indicate that the ranges of effectiveness to the different fungicides are variable for *B. cinerea* isolates.

In the germination and conidial production of *B. cinerea*, pyrimethanil and fenpyrazamine were less effective than the other fungicides. In particular, fenpyrazamine, which showed a low EC_{50} value of 0.9 μg/ml for the inhibition of mycelial growth, was the least effective against both the germination and conidial production of *B. cinerea*. The pyrazole fungicide fenpyrazamine was effective for inhibiting mycelial growth, although it was not effective at a high concentration (EC_{50} = 0.9 μg/ml and EC_{90} > 3,000 μg/ml) compared with the other fungicides. This different effect of fenpyrazamine is due to its different action on the mycelial growth, germination, and conidiation of *B. cinerea*. Fenpyrazamine is a new active substance, discovered and developed by Sumitomo Chemical Company, which was registered in South Korea in April 2012. Its recent development makes it necessary to study its mode of action at the molecular level during fungal development.

In conclusion, we showed the effectiveness of different classes of fungicide on the growth, germination, and conidiation of *B. cinerea*. However, our results are based only on a *B. cinerea* isolate from Chuncheon, Kangwon Province; therefore, it is necessary to collect many *B. cinerea* isolates and evaluate the sensitivity and resistance of *B. cinerea* isolates to the fungicides. Our results provide basic information on the chemical management of *B. cinerea* and are expected to contribute to future work.

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