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PLANT PROTECTION - Article

Resistance of *Botrytis cinerea* to fungicides controlling gray mold on strawberry in Brazil

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ABSTRACT: The aim of this study was to evaluate the resistance of *Botrytis cinerea* to the fungicides currently used for its control in Brazil. Isolates of the fungus were collected from different strawberry-producing fields in the states of Espírito Santo, Minas Gerais, and São Paulo, Brazil. First, a total of 183 isolates were identified at the species level using specific primers for the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene. The isolates were grown on potato dextrose agar (PDA) containing the fungicides procymidone, iprodione, and thiophanate-methyl in different concentrations: 0.0 (control), 0.1; 1.0; 10.0; 100.0 and 1,000.0 $\mu g \cdot mL^{-1}$. The percentage of mycelial growth inhibition was used to determine the effective concentration of the fungicide that was able to inhibit colony growth by 50% (EC_{50}). Approximately 25.7% of the isolates were resistant to iprodione, 53.0% were resistant to procymidone, and 93.0% were resistant to thiophanate-methyl. Moreover, cross-resistance and multiple resistance were verified, with 19.7% of the isolates showing resistance to 3 fungicides simultaneously. This finding explains the ineffectiveness of fungicides application to control gray mold in strawberry fields in Brazil and highlights the need for new strategies to manage this disease in the culture.

Key words: *Fragaria x ananassa*, chemical control, dicarboxamides, methyl benzimidazole carbamate.

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INTRODUCTION

Gray mold caused by the fungus *Botrytis cinerea* Pers. ex Fries is a very common disease, affecting approximately 200 species of different botanical families, among them fruits, vegetables, ornamental, and wild plants (Elad et al. 2007; Williamson et al. 2007). The fungus presents high adaptability, occurring in different areas such as tropical, subtropical, temperate dry and wet, and causes severe damage (Elad et al. 2007).

Gray mold is considered the main disease in strawberry (*Fragaria* x *Ananassa*) production (Maas 1998; Costa et al. 2014) because the fungus can infect leaves, stems, flowers, and fruits at any stage of development. Infected organs become necrotic, with an intense sporulation, and a gray color, which characterizes the disease. Although the fungus can infect all parts of the plant, the damage is more common during the production phase when the pathogen infects flowers (Bristow et al. 1986; Maas 1998; Elad et al. 2007; Costa et al. 2014).

Chemical control by fungicides is one of the most important measures to control gray mold worldwide. In Brazil, under favorable weather conditions, several applications are required during a culture cycle (Kimati et al. 1997; Costa et al. 2014). Currently, only 3 groups of fungicides are registered by the Ministério da Agricultura, Pecuária e Abastecimento (MAPA) for strawberry gray mold control in Brazil: Dicarboxamides, Methyl Benzimidazole Carbamate (MBC), and Anilinopyrimidine (Brasil 2016).

Although chemical control is the main strategy to control gray mold, the emergence of fungal populations resistant to fungicides has been a great challenge for strawberry growers (Brent and Hollomon 2007b). Due to the loss of efficiency of the fungicides, producers tend to increase their use to achieve an effective control of the disease (Zambolim et al. 2007). Several studies have reported cases of resistance of *B. cinerea* to fungicides of the dicarboxamide and MBC groups. Yourman and Jeffers (1999) investigated the sensitivity of *B. cinerea* isolates in ornamental plantations and showed that more than 80% of the isolates analyzed were resistant to thiophanate-methyl fungicide. Furthermore, the occurrence of multi-resistance to dicarboxamides and benzimidazoles has been demonstrated. LaMondia and Douglas (1997) found that 70% of *B. cinerea* isolates obtained from a greenhouse were resistant to benomyl and thiophanate-methyl fungicides, while approximately 35% of the isolates were resistant to iprodione fungicide. Isolates of *B. cinerea* obtained from pear also showed resistance to iprodione and thiabendazole fungicides (Lennox and Spotts 2003). Resistance of this fungus to dicarboxamides and MBC fungicides is common, and there are reported cases of isolates resistant to thiophanate-methyl, pyraclostrobin, boscalid, cyprodinil, fenhexamid, iprodione, and fludioxonil fungicides, simultaneously (Fernández-Ortuño et al. 2014). Although various mechanisms by which *B. cinerea* becomes resistant to fungicides have been identified, the most common is the emergence of mutations in the target sites of fungicides (Leroux et al. 2002).

Understanding the profile of pathogen populations in cultivated areas is one of the first steps for an integrated management of this disease. In Brazil, little is known on the resistance of *B. cinerea* populations to fungicides registered for strawberry crop. Therefore, the goal of this study was to evaluate the resistance of *B. cinerea* isolates obtained from different strawberry-producing fields in Brazil to fungicides most widely used for its control. This information is important to help in the development of strategies aiming to improve disease control and prevent the loss of fungicide efficiency.

MATERIAL AND METHODS

Obtaining the isolates

The isolates were obtained from strawberry plants showing symptoms of gray mold in commercial plantations in 18 different cities in the states of Espírito Santo (ES), Minas Gerais (MG), and São Paulo (SP), Brazil, during 2012, totaling 183 isolates (Table 1).

In each field, a sample of leaf, flower or fruit was collected and placed in labeled plastic bags. To obtain monosporic isolates, conidia were collected from the surface of infected material and placed in 1 mL of sterile distilled water containing 1% Tween 60. Approximately 200 µL of conidial suspension were spread in a Petri dish containing water-agar medium and streptomycin (100 $\mu g \cdot mL^{-1}$). After incubation at 22 °C for 24 – 36 h, 1 germinated conidia was collected and transferred to potato dextrose agar (PDA) medium. All isolates were stored in silica and maintained at 5 °C.

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Table 1. Number of isolates collected in different cities in the states of Espírito Santo, Minas Gerais, and São Paulo.

City	State	Number of isolates
Domingos Martins	ES	5*
Santa Maria do Jetibá	ES	4
Venda Nova do Imigrante	ES	7
Alfredo Vasconcelos	MG	39
Barbacena	MG	4
Ressaquinha	MG	19
Carandaí	MG	3
Senador Amaral	MG	29
Bom Repouso	MG	29
Pouso Alegre	MG	10
Estiva	MG	13
Cambuí	MG	4
Coimbra	MG	2
Ervália	MG	1
Viçosa	MG	1
São Miguel do Anta	MG	4
Atibaia	SP	4
Jarinu	SP	5

*Each isolate was obtained from a different field. ES = Espírito Santo; MG = Minas Gerais; SP = São Paulo.

Survey concerning the fungicides used to control gray mold of strawberry

During the collection of isolates in the different producing areas, a survey was conducted on fungicide use. This information was obtained from 121 areas of commercial cultivation in the 3 states listed above.

Molecular identification of the isolates

Genomic DNA was extracted from all isolates, quantified using a spectrophotometer, and diluted to a final concentration of 50 ng·μL^{-1}. Species identification was performed by polymerase chain reaction (PCR) using primers that targeted partial sequences of the glyceraldehyde-3-phosphate dehydrogenase gene (G3PDH), G3PDH-F1 (5'- GGACCCGAGCTAATTTATGTCACGT-3'), and G3PDH-F2 (5'-GGGTGTCAACAACGAGACCTACACT-3'), specific to *B. caroliniana* and *B. cinerea*, respectively. These primers were combined with the reverse primer G3PDH-R (5'-ACCGGTGCTCGATGGGATGAT -3') (Li et al. 2012a). The amplification conditions were considered according to Li et al. (2012a). Isolates of *B. cinerea* (CA25, WM6, and CB17) and *B. caroliniana* (CA3, WM4, CB15, and BC11HP33) (Li et al. 2012a; Li et al. 2012b), which were characterized in previous studies, were used as positive controls.

Sensitivity of the isolates to fungicides

To obtain different concentrations of the active ingredients (a.i.) of the fungicides iprodione, procymidone, and thiophanate-methyl, commercial products were used: Rovral SC (500 g·L^{-1} iprodione), Sumilex 500 WP (500 g·L^{-1} procymidone), and Cercobin 700 WP (500 g·L^{-1} thiophanate-methyl). These fungicides were selected because they are the most widely used in strawberry crops to control gray mold, based on our previous survey (Table 2).

Table 2. Survey concerning the use of fungicides in different areas from where the isolates used in this study were obtained.

Description	Number of areas
Total of visited areas to obtain the isolates	183
Areas where information on the use of fungicides could be obtained	121
Areas with application of at least 1 fungicide	107
Procymidone application	68
Thiophanate-methyl application	38
Iprodione application	36
Iprodione and procymidone application	29
Procymidone and thiophanate-methyl application	25
Iprodione and thiophanate-methyl application	12
Thiophanate-methyl, iprodione, and procymidone application	12

Each fungicide was added to PDA medium at concentrations of 0.0 (control), 0.1; 1.0; 10.0; 100.0 and 1,000.0 μg·mL^{-1}. Then, a 7-mm diameter mycelial plug (obtained from 3-day-old colonies grown on PDA) was placed at the center of Petri dishes containing 8 mL of PDA + fungicide at different concentrations. For each concentration of fungicide, 3 Petri dishes were used, accounting for 3 replicates. The Petri dishes were maintained at 22 °C in the dark. After the second day of growth, the diameter of the colony was measured in 2 perpendicular directions with the aid of a digital caliper (Metrotools - 150 mm). Using the control treatment (0.0 μg·mL^{-1} of fungicide) as standard, the percentage of growth at each concentration was obtained. This value was subtracted from 100 to yield the percentage of growth inhibition at each dose of the fungicide. The effective concentration (μg·mL^{-1}) of the fungicide that was able to inhibit mycelial growth by 50% (EC_{50}) was calculated for each isolate by linear regression of the mycelial growth inhibition *versus* the log10 transformation of the fungicide concentration using the software SAS 9.0 (SAS Institute Inc, Cary, NC, USA).

Data analysis

To discriminate the isolates that were sensitive or resistant to the fungicides, we considered previous studies in which this relationship had been well defined. For fungicides of the dicarboxamide group (iprodione and procymidone), the discriminatory dose was 1 μg·mL^{-1} (LaMondia and Douglas 1997; Myresiotis et al. 2007) compared with 10.0 μg·mL^{-1} for the MBC fungicide (thiophanate-methyl) (LaMondia and Douglas 1997; Lennox and Spotts 2003). Then, the isolates were classified as resistant to dicarboxamide fungicides (iprodione and procymidone) if $EC_{50} > 1.0$ and resistant to MBC (thiophanate-methyl) when $EC_{50} > 10.0$.

A Pearson correlation analysis between the sensitivity to iprodione and procymidone was performed to check the existence of cross-resistance. Similarly, the correlation between the sensitivity to iprodione or procymidone and thiophanate-methyl was evaluated to determine the existence of multi-resistance. These tests were performed at 5% of significance using the software SAS 9.0 (SAS Institute Inc, Cary, NC, USA).

RESULTS AND DISCUSSION

Among the fungicides registered by the MAPA to control gray mold on strawberry in Brazil (Brasil 2016), the most commonly used are those belonging to the chemical group of dicarboxamides (procymidone and iprodione) (Table 2). In our survey, of the 121 areas of commercial cultivation where information on the use of fungicide could be obtained, in 107 areas the application of at least 1 fungicide was confirmed. In 64.0% of these areas, the fungicide procymidone was used, whereas in 36.0% it was used iprodione. Thiophanate-methyl, belonging to the MBC group, was used in 38.0% of the analyzed areas. Moreover, the simultaneous application of 2 or 3 fungicides was commonly observed.

The isolates showed high variability when grown on PDA medium at 22 °C. Due to these morphological variations, we suspected that the isolates would correspond to different species of *Botrytis*. However, the analysis of the G3PDH gene fragment, by PCR using specific primers, revealed the presence of a fragment of 238 bp for all isolates, which corresponded to *B. cinerea*.

Most isolates of *B. cinerea* (74.3%) were sensitive to the iprodione fungicide, and 31.0% had EC_{50} values < 0.1 μg·mL^{-1}. The remaining isolates (25.7%) had EC_{50} values > 1.0 μg·mL^{-1} and were resistant to the fungicide, although the EC_{50} values did not exceed 10.0 μg·mL^{-1} (Figure 1). In the case of procymidone, 47.0% of the isolates were sensitive, and 13.1% had EC_{50} values < 0.1 μg·mL^{-1}. The remaining isolates (53.0%) were resistant (EC_{50} > 1.0 μg·mL^{-1}), and 5.0% had EC_{50} values that exceeded 1,000 μg·mL^{-1}. On the other hand, when assessing the sensitivity of the isolates to the fungicide thiophanate-methyl, a very different behavior was observed, i.e. 93.0% of the isolates were resistant (EC_{50} values > 10.0 μg·mL^{-1}), and 73.0% had EC_{50} values greater than 1,000 μg·mL^{-1}.

Based on the responses of sensitivity and resistance to procymidone, iprodione, and thiophanate-methyl, the isolates were grouped into 8 different phenotypes (Figure 2). Only 4.9% of the isolates were fully sensitive to all 3 fungicides, whereas 19.7% were resistant to all fungicides simultaneously. Regarding the resistance to only 1 fungicide, procymidone was the one with lowest percentage of resistance (1.6%), followed by iprodione (14.8%) and thiophanate-methyl (22.4%). Multiple resistance (between different chemical groups) to thiophanate-methyl and procymidone was observed in 17.5% of the isolates, and, to thiophanate-methyl and iprodione, in 14.8% of the isolates. The cross-resistance between iprodione and procymidone was observed in 4.4% of the isolates. Positive and significant correlations ($p < 0.05$) were observed between the resistance to procymidone and iprodione ($r = 0.278$; p-value = 0.000140) and between thiophanate-methyl and iprodione ($r = 0.169$; p-value = 0.0220). On the other hand, there was no correlation between the resistance to thiophanate-methyl and procymidone ($r = 0.0997$; p-value = 0.179).

In ES, most isolates were resistant to thiophanate-methyl (81.3%) and procymidone (68.8%), while only 25.0% of the isolates were resistant to iprodione (Table 3).

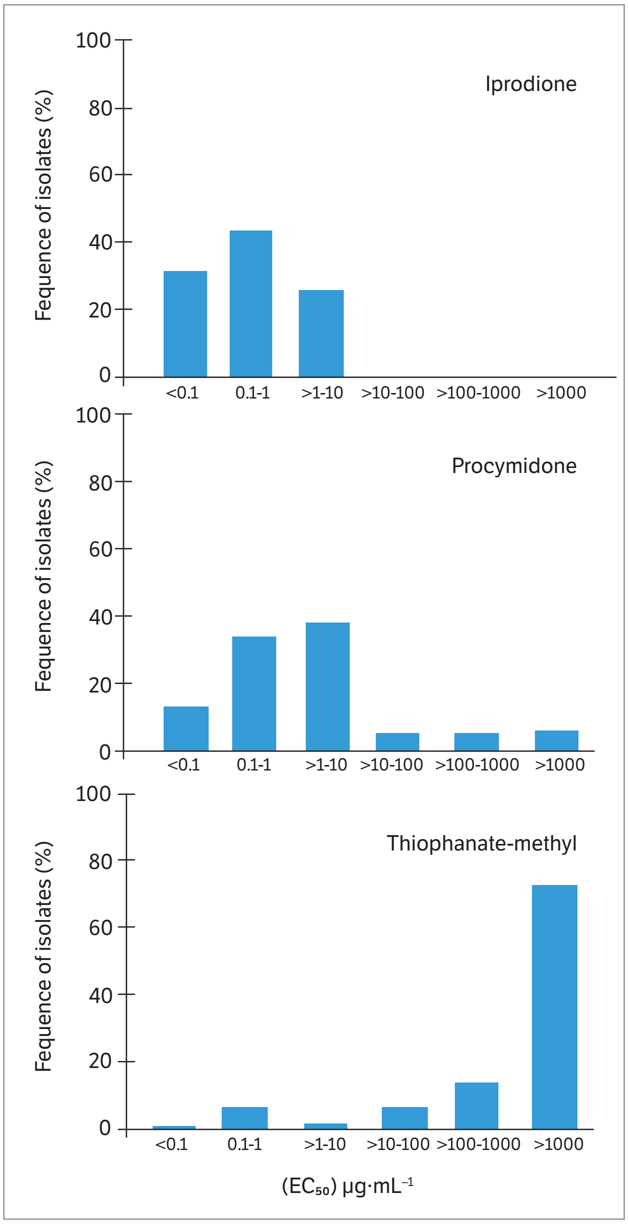

Figure 1. Frequency distribution of the effective concentration values that inhibit mycelial growth by 50% (EC_{50}) for the different isolates grown in medium containing iprodione, procymidone or thiophanate-methyl fungicides.

The analysis of the isolates from MG showed that 93.7% were resistant to thiophanate-methyl, 49.0% were resistant to procymidone, and 27.2% were resistant to iprodione. All isolates from SP were resistant to thiophanate-methyl and sensitive to iprodione, while 89.0% were resistant to procymidone. In general, the lowest percentage of resistance was observed for the iprodione fungicide, particularly in SP, where resistant isolates were not detected. Therefore, its use to control gray mold on strawberry crops should be prioritized over the other fungicides, especially thiophanate-methyl.

Botrytis cinerea belongs to a group referred to as "high risk" for the development of fungicide resistance due to its high reproductive capacity, facility of dispersion, and wide host range (Brent and Hollomon 2007a). Additionally, this fungus presents a high genetic variability that is

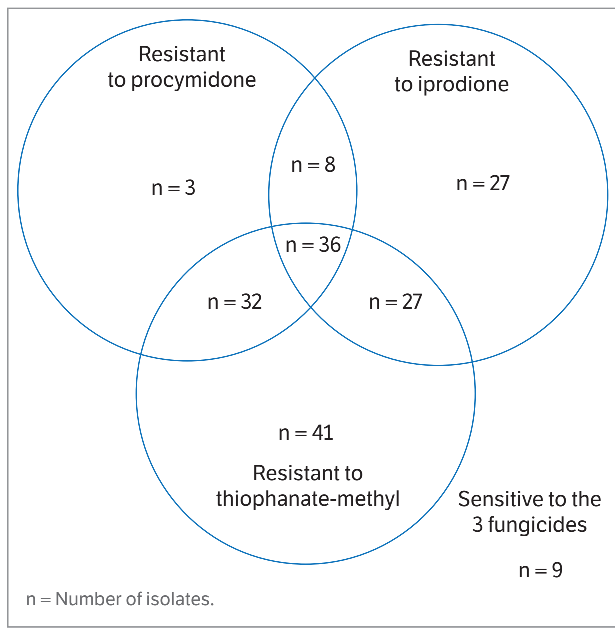

Figure 2. Number of isolates showing the phenotypes that are sensitive and resistant to iprodione, procymidone, and thiophanate-methyl fungicides.

Table 3. Percentage of isolates from the states of Espírito Santo, Minas Gerais, and São Paulo showing resistance to the fungicides thiophanate-methyl, procymidone, and iprodione.

State	Number of isolates	Resistance (%)		
		Thiophanate-methyl	Procymidone	Iprodione
Espírito Santo	16	81.3 (13)*	68.8 (11)	25.0 (4)
Minas Gerais	158	93.7 (148)	49.0 (78)	27.2 (43)
São Paulo	9	100.0 (9)	89.0 (8)	0.0 (0)
Total	183	93.0 (170)	53.0 (97)	25.7 (47)

*In parenthesis: number of isolates.

maximized by the presence of transposable elements in the genome (Samuel et al. 2012). Moreover, the use of large amounts of fungicide in strawberry plantations has contributed to increase the occurrence of resistance of *B. cinerea* to fungicides. In this study, it was observed a high resistance of *B. cinerea* population to iprodione, procymidone, and thiophanate-methyl, which are the fungicides most commonly used in strawberry fields in Brazil.

Despite the lower use of thiophanate-methyl in the analyzed areas compared with dicarboxamides, a high percentage of resistance to thiophanate-methyl was observed in the current study (93.0% of the isolates). It was also verified by Yourman and Jeffers (1999), who reported that more than 80.0% of the isolates were resistant to this fungicide. The resistance of *B. cinerea* to fungicides of the MBC group is very common, as observed in several studies (Moorman and Lease 1992; LaMondia and Douglas 1997; Lennox and Spotts 2003; Myresiotis et al. 2007; Yoon et al. 2008; Fernández-Ortuño et al. 2014). This resistance has been shown to persist in the population for many years even after the discontinued use of the fungicides (Walker et al. 2013).

The analyzed population was more sensitive to iprodione and procymidone compared with thiophanate-methyl. Despite this, there were isolates with resistance to iprodione and procymidone, including cases of cross-resistance between both fungicides. Grabke et al. (2014) analyzed *B. cinerea* populations in strawberry plantations located in Florida, North Carolina, and South Carolina and also observed isolates resistant to these fungicides. LaMondia and Douglas (1997) studied the resistance of *B. cinerea* to MBC (benomyl and thiophanate-methyl) and dicarboxamide (iprodione) and observed that 70.0% of the isolates were resistant to the fungicides of the first group and approximately 35.0% were resistant to the second group. In addition, there were cases of cross-resistance, which is similar to our findings.

Multiple resistance to the fungicides of the dicarboxamide and MBC groups was observed in the analyzed population, with 17.5% of the isolates resistant to thiophanate-methyl and procymidone, 14.8% resistant to thiophanate-methyl and iprodione, and 19.7% resistant to the 3 fungicides simultaneously. Although these data are alarming, they are consistent with other studies that have reported the simultaneous resistance of *B. cinerea* isolates to thiophanate-methyl, pyraclostrobin, boscalid, cyprodinil, fenhexamid, iprodione, and fludioxonil fungicides (Amiri et al. 2013; Fernández-Ortuño et al. 2014).

Our research has raised a serious concern in strawberry crop production in Brazil regarding the resistance of *B. cinerea* to the 3 fungicides most widely used to control gray mold. The high levels of resistance observed with thiophanate-methyl and the high number of isolates with resistance to the 3 fungicides are alarming data. Taken together, the information generated from this study will be helpful for the development of strategies aiming to improve disease control and will lead to an efficient and sustainable use of fungicides in strawberry culture.

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