Plant Disease • 2018 • 102:1803-1810 • https://doi.org/10.1094/PDIS-08-17-1221-RE

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Sensitivity of *Botrytis cinerea* Isolates from Conventional and Organic Strawberry Fields in Brazil to Azoxystrobin, Iprodione, Pyrimethanil, and Thiophanate-Methyl

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Abstract

Botrytis fruit rot, caused by *Botrytis cinerea*, is one of the most important strawberry diseases worldwide, and fungicide applications are often used to manage the disease in commercial production. Isolates of *B. cinerea* were collected from conventional and organic strawberry fields in four Brazilian States from 2013 to 2015 and their sensitivity to the main single-site mode-of action fungicides used in Brazil was tested. Resistance to azoxystrobin, iprodione, pyrimethanil, and thiophanate-methyl was found and values for effective concentration that inhibited mycelial growth by 50% were higher than 71.9, 1.2, 5.0, and 688 μg/ml, respectively, regardless the production system. Resistance to these fungicides was observed in 87.5, 76.6, 23.4, and 92.2% of isolates from conventional fields and 31.4, 22.9, 14.3, and 51.4% of isolates from organic fields, respectively. Moreover, frequencies of isolates with multiple fungicide resistance to the four active ingredients were 20.6 and 2.8% whereas 6.3 and 27.8% were sensitive to the four fungicides for conventional and organic areas, respectively. Molecular analyses of the cytochrome *b*, β-tubulin, and *bos1* genes revealed the presence of G143A; E198A; and I365 N/S, Q369P, or N373S mutations, respectively, in resistant isolates of *B. cinerea*. Field rates of fungicides sprayed preventively to inoculated strawberry fruit failed to control disease caused by the respective resistant isolates.

Botrytis fruit rot (BFR), caused by *Botrytis cinerea* Pers., is one of the most important diseases in strawberry fields worldwide, and is responsible for severe pre- and postharvest losses due to infection of flowers and fruit (Fernández-Ortuño et al. 2016; Mertely et al. 2000). *B. cinerea* is a necrotrophic pathogen that can overwinter as mycelia, spores, or sclerotia in crop debris (Braun and Sutton 1987; Mertely et al. 2000). Spores are usually spread by air and water from rain or overhead irrigation, and may infect strawberry plants, especially via flowers but also fruit through surface injuries (Droby and Lichter 2007). Rot symptoms develop soon after infection or infections may remain quiescent until fruit maturity or after harvest (McNicol et al. 1985). Symptoms appear as brown lesions that can develop from small to large sizes and become covered with mycelium and spores of the pathogen, rendering the fruit dry and tough with little or no leakage of fruit content (Droby and Lichter 2007). Favorable environmental conditions for infection and symptom development are temperatures between 20 to 23°C and prolonged periods of high humidity or rain (Wilcox and Seem 1994).

BFR is usually managed by a combination of cultural practices, use of less susceptible cultivars, and chemical control. The use of healthy nursery transplants is usually the first recommended measure because propagation material may carry quiescent infections and become the primary source of inoculum of *B. cinerea* in an area (Oliveira et al. 2017). Some strawberry cultivars are less susceptible to BFR but there is no complete resistance (Legard et al. 2000; Seijo et al. 2008). Cultural practices such as use of drip irrigation instead of overhead irrigation, frequent harvest of fruit, removal of diseased and unmarketable fruit, and removal of crop residue and senescent foliage are also recommended for control of the disease (Droby and Lichter 2007; Mertely et al. 2000). However, preventive applications of fungicide are traditionally the standard and most effective control method in conventional production (Amiri et al. 2013; Mertely et al. 2002). In organic production systems, growers rely on disease-free plant material, use of less-susceptible cultivars, weed management, use of protected structures such as low tunnels, crop rotation, beneficial organisms, and plant extracts (biological control).

Chemical control of BFR relies on application of single- and multisite mode-of-action fungicides throughout the strawberry season, especially during bloom and fruiting (Amiri et al. 2013; Mertely et al. 2002). Single-site products registered on strawberry in Brazil include quinone-outside inhibitors (QoI), dicarboximides (DC), demethylation inhibitors, aniline-pyrimidines (AP), and methyl benzimidazole carbamates (MBC) (AGROFIT 2017). The MBC thiophanate-methyl, the DCs iprodione and procimidone, and the AP pyrimethanil are labeled for BFR control. Although the QoI azoxystrobin is labeled for Mycosphaerella leaf spot management, it also suppresses BFR, and it is on the list of pesticides permitted in the "Integrated Production of Strawberry" program in Brazil (IDAF 2012). Some of the active ingredients are registered as single fungicides, whereas others are also combined in prepackaged mixtures such as iprodione + pyrimethanil and azoxystrobin + difenoconazole (AGROFIT 2017). Organic growers in Brazil commonly use plant extracts; lime, copper, and sulfur compounds; essential, mineral, and vegetable oils; as well as Bordeaux mixture as inorganic multisite mode-of-action fungicides to manage diseases of strawberry (MAPA 2017).

Because chemical control has been widely and regularly used in strawberry production, selection of fungicide-resistant mutants that are no longer controlled by fungicides may put the control of BFR at risk (Fernández-Ortuño et al. 2016; Ishii et al. 2009; Rupp et al. 2017). Site-specific fungicides such as QoI, DC, and MBC are classified as having a high risk of resistance development, whereas AP are classified as medium risk (FRAC 2013). The most common

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Present address of J. S. Baggio: Gulf Coast Research and Education Center, University of Florida

Funding: This research was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) under contract numbers 2013/21930-6 and 2014/16198-7.

*The *e*-Xtra logo stands for "electronic extra" and indicates that one supplementary figure is published online.

Accepted for publication 11 March 2018.

mechanism by which pathogens can become resistant to fungicides is the alteration of the target site (Deising et al. 2008; FRAC 2017). In fact, emergence of *B. cinerea* resistance to site-specific fungicides has been extensively reported in several countries (Amiri et al. 2013; Banno et al. 2009; Fernández-Ortuño et al. 2016; Rupp et al. 2017).

QoI fungicides act by inhibiting ATP production during mitochondrial respiration by binding at cytochrome *b* and blocking electron transfer (Bartlett et al. 2002). Resistance to QoI may occur as mutations in the cytochrome *b* (*cytb*) gene, with amino acid replacements at codons 129, 137, and 143, and has been widely reported in *B. cinerea* populations of strawberry (Amiri et al. 2013; Fernández-Ortuño et al. 2016). The G143A mutation confers high levels of resistance and is the most commonly found in field isolates, whereas the mutations F129L and G137R are associated with moderate levels of resistance (Bartlett et al. 2002).

The mode of action of DC fungicides consists of interfering with the osmotic signal transduction pathway (Yamaguchi and Fujimura 2005). Resistance to DC, already reported for *B. cinerea* from strawberry, is usually related to point mutations in the *bos1* gene, and the most common mutations in reduced-sensitive or resistant isolates of *B. cinerea* are I365S/N, Q369P, and N373S (Banno et al. 2008; Fernández-Ortuño et al. 2016; Ma et al. 2007).

AP fungicides target the synthesis of methionine and other amino acids and inhibit the secretion of hydrolytic enzymes that are essential during pathogen infection (Masner et al. 1994). Resistance to AP fungicides has been reported for *B. cinerea* of strawberry, vineyards, and vegetables worldwide (Amiri et al. 2013; Leroux et al. 1999; Myresiotis et al. 2007). However, the molecular mechanisms of resistance in plant pathogens have not yet been determined.

MBC fungicides inhibit nuclear division by binding to β-tubulin (β-*tub*) and disrupting chromosome segregation and migration (Davidse 1986). Resistance to MBC fungicides is usually associated with the point mutations F167Y, E198A/K, and F200Y in the β-*tub* gene (Ma and Michailides 2005). *B. cinerea* populations resistant to MBC have been reported worldwide (Banno et al. 2008; Fernández-Ortuño et al. 2015; Rupp et al. 2017).

Conventional strawberry production demands a large number of pesticide sprays to control insects, mites, and diseases: it is estimated that up to 30 to 40 fungicide applications are made per season in production areas in Brazil (Zambolim and Costa 2006). In addition to the risk of fungicide resistance, the presence of pesticide residue on food and in the environment has raised concern about the indiscriminate use of pesticides in agriculture. Recent reports by the Brazilian National Health Surveillance Agency (ANVISA 2016) have shown that 72% of strawberry samples analyzed contained residues of pesticides not registered for strawberry or levels above the permitted limit (ANVISA 2016). Thus, public concern about sustainable food production has led to an increased demand for organic products and, as a result, growth of the organic market and multiplication of farms adopting organic practices (Andow et al. 2017).

Considering the presence of fungicide resistance worldwide, monitoring of conventional and organic strawberry production fields is important to develop a resistance management program for growers in Brazil. Therefore, this study was intended to (i) evaluate the sensitivity to azoxystrobin, iprodione, pyrimethanil, and thiophanate-methyl in *B. cinerea* isolates collected from conventional and organic strawberry fields in Brazil through mycelial growth, spore germination, and fruit assays; and (ii) molecularly characterize resistance mechanisms in isolates with different levels of sensitivity to azoxystrobin, iprodione, and thiophanate-methyl.

Materials and Methods

Fungal isolates and culture. In total, 99 *B. cinerea* isolates were collected from conventional and organic strawberry fields from 2013 to 2015 in four Brazilian States: São Paulo, Minas Gerais, Espírito Santo, and Bahia. In 2013, 27 and 19 isolates were collected; in 2014, 34 and 11 isolates were collected; and, in 2015, 3 and 5 isolates were collected from conventional and organic areas, respectively. Pathogens were isolated from symptomatic flowers and fruit by transferring spores from the lesions to water agar. After 2 to 3 days, single-spore isolates of *B. cinerea* were transferred to malt-yeast-agar medium (MYA). Isolates were grown for 10 to 14 days at 23°C under constant light, preserved on filter paper, and stored dry on filter paper at −20°C.

Fungicides. Commercial formulations of azoxystrobin (Abound Flowable and Amistar WG, Syngenta Crop Protection), iprodione (Rovral Brand 4 Flowable and Rovral SC, FMC; Bayer Crop Science), pyrimethanil (Scala Brand SC; Bayer Crop Science), and thiophanate-methyl (Cercobin 700 WP, Ihara and Topsin 4.5 FL; UPI) were used for fungicide sensitivity trials. For in vitro assays, salicylhydroxamic acid (SHAM, 99% active ingredient; Sigma-Aldrich), which inhibits the fungal alternative respiration pathway, was dissolved in methanol and added to azoxystrobin-amended medium at a final concentration of 100 μg/ml (Amiri et al. 2013).

Fungicide sensitivity in mycelial growth assays. Mycelial growth sensitivity was evaluated using the spiral gradient dilution method (Amiri et al. 2013; Förster et al. 2004). MYA medium (50 ml) containing agar at 20 g/liter ($MYAA^+$) was poured into 150-mm-diameter plates to produce the inoculum strips (Amiri et al. 2013). A spore suspension (1 ml, 10^6 spores/ml) of each isolate was spread onto the $MYAA^+$ medium and, after 48 h at 23°C, an agar slicer was used to cut the strips. Stock suspensions of azoxystrobin at 9,304.1 μg/ml, iprodione at 3,476.2 μg/ml, pyrimethanil at 4,282.34 μg/ml, and thiophanate-methyl at 1,760.1 and 88,005.2 μg/ml were prepared in sterile distilled water, according to concentrations predetermined by spiral gradient endpoint software (Förster et al. 2004). Each fungicide stock suspension was applied spirally to the surface of 150-mm-diameter plates containing potato dextrose agar (PDA) or CzapeK-Dox agar (CzA) media using a spiral plater (Autoplate 4000 and 5000 models; Spiral Biotech, Inc.). PDA was used to evaluate the sensitivity to azoxystrobin, iprodione, and thiophanate-methyl, whereas CzA was used for pyrimethanil. After fungicide application with a spiral plater, these stock concentrations resulted in actual fungicide concentration ranges in the agar of 0.35 to 71.9 μg/ml for azoxystrobin, 0.15 to 27.2 μg/ml for iprodione, 0.27 to 33.6 μg/ml for pyrimethanil, and 0.07 to 13.8 and 3.6 to 688 μg/ml for thiophanate-methyl. Application of inoculum strips and evaluation of mycelial growth were made following the method of Amiri et al. (2013). Each fungicide–isolate combination was replicated three times and experiments were performed twice.

Fungicide sensitivity in spore germination assays. Because spore germination is the stage of fungi development most sensitive to QoI fungicides and has been used in other studies (Chatzidimopoulos et al. 2013; Fernández-Ortuño et al. 2012), sensitivity to azoxystrobin was tested in a spore germination assay for 15 isolates. Spore suspensions (1×10^5 ml^{-1}) were prepared in sterile distilled water from 14-day-old cultures. Three aliquots (30 μl each) of spore suspension were placed on malt-extract-agar plates (10 g of malt extract and 15 g of agar in 1.0 liter of distilled water) amended with SHAM (100 μg/ml) and azoxystrobin at 0, 0.1, 0.5, 1, 5, 10, or 100 μg/ml. Three plates (replications) per treatment were used and experiments were performed twice. Spore germination was evaluated after 16 to 24 h at 23°C under constant light and spores with a germ tube longer than the spore diameter were considered germinated. The germination percentage was determined by counting 100 random spores under an optical microscope (×400).

Fungicide sensitivity in fruit assays. To evaluate the occurrence of practical resistance, fungicide-treated strawberry fruit were inoculated with isolates with different levels of sensitivity. Immature 'Florida 127' fruit were harvested from experimental plots at the Gulf Coast Research and Education Center at the University of Florida, Wimauma. In the laboratory, the sepals were removed and fruit were washed in 0.05% sodium hypochlorite for 2 min, then rinsed twice in distilled water. Six strawberry fruit per isolate were placed in egg trays inside plastic boxes, wounded using a 4-mm cork-borer to a depth of 4 mm, and sprayed with azoxystrobin (80 μg/ml), iprodione (750 μg/ml), pyrimethanil (600 μg/ml), and thiophanate-methyl (490 μg/ml) at recommended field rates in Brazil, using a spray

bottle. Controls were sprayed with sterile distilled water. Fruit were incubated for 24 h at 23°C, then inoculated on the wound with 40 µl of each isolate spore suspension at 10^6 spores/ml. Inoculated fruit were kept in sealed humidity chambers at 23°C and disease incidence and severity were assessed 5 days after inoculation. For each isolate–fungicide combination, three replicate boxes were used and the experiments were conducted twice. Disease incidence was determined as the percentage of fruit showing symptoms based on the total inoculated, and disease severity was calculated as the area (proportion) of the visible part of each fruit that became infected.

Data analysis. Data from mycelial growth, spore germination, and fruit assays from repeated experiments were combined after Levene's test indicated that variances were homogeneous. Linear regressions of spore germination on the $\log_{10}$-transformed fungicide concentrations were used to estimate values for effective concentration that inhibited mycelial growth by 50% (EC_{50}). Disease incidence and severity data from fruit assays were subjected to an analysis of variance and means were separated using Fisher's least significant difference test. Statistical analyses were performed using the Statistica 7.0 (Statsoft) and SAS (version 9.2; SAS Institute Inc.) software.

Characterization of mutations linked to resistance to QoI, DC, and MBC fungicides. *B. cinerea* isolates were cultured on MYA medium for 7 days and mycelium and spores were collected to perform DNA extractions. Genomic DNA was extracted using the FastDNAKit (MP Biomedicals, LLC), according to the manufacturer's protocol.

Analysis of cytb *gene sequences linked to resistance to QoI fungicides.* The partial *cytb* gene from all *B. cinerea* isolates was amplified using primers Qo13ext (5′-GGTATAACCCGACGGGGT TATAGAATAG-3′) and Qo14ext (5′-AACCATCTCCATCCACC ATACCTACAAA-3′) (Leroux et al. 2010). Polymerase chain reactions (PCR) were performed in a final volume of 25 µl containing 3 µl of 7.5 mM $MgCl_2$ 5× buffer (Phire Hot Start II; Thermo Scientific), 2 µl of 25 mM $MgCl_2$, 1 µl of 5 M betaine (Sigma-Aldrich), 1 µl of 10 mM dNTP (Promega Corp.), 1.5 µl of each 10 µM primer, 0.1 µl of 0.5 U of Taq polymerase (Phire Hot Start II; Thermo Scientific), and 2 µl of DNA at 75 ng/µl. PCR was performed using the following conditions: an initial denaturation at 95°C for 3 min; 40 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 5 min. PCR products were visualized under UV-light in a 1% agarose gel in 1× Tris-acetate-EDTA buffer (0.04 M Tris-acetate and 0.0001 M EDTA) with ethidium-bromide staining at 100 V for 25 min.

A PCR restriction fragment length polymorphism method was used for rapid detection of the G143A mutation in *B. cinerea* isolates (Fernández-Ortuño et al. 2012). PCR products of 22 isolates were purified with the ExoSAP-IT PCR purification kit (Affymetrix, Inc.), according to the manufacturer's instructions, and sent to Genewiz Corporation for sequencing in both directions. Sequences were assembled, translated, and aligned using MEGA (ver. 6) and BioEdit (ver. 7.2.5.), and analyzed by BlastN against the GenBank database.

Analysis of bos1 *gene sequences linked to resistance to DC fungicides.* The partial *bos1* gene from 14 *B. cinerea* isolates was amplified using the primer pair BF2 (5′-CAACGTTATGGCACA AAATCTCA-3′) and BR2 (5′-AAGTTTCTGGCCATGGTGTTCA-3′) (Ma et al. 2007). PCR was conducted in a final volume of 25 µl containing 2.5 µl of ThermoPol Buffer (BioLabs), 0.5 µl of 10 mM dNTP (Promega Corp.), 1 µl of each 10 µM primer, 0.125 µl of Taq DNA polymerase (5,000 U/ml; BioLabs), and 2 µl of DNA at 25 ng/µl. The following parameters were used: an initial denaturation at 95°C for 3 min; 40 cycles of 94°C for 1 min, 61°C for 1 min, and 72°C for 1.5 min; and a final extension at 72°C for 5 min. PCR products were separated, purified, sequenced, and analyzed as described for the *cytb* gene.

Analysis of β-tub *gene sequences linked to resistance to MBC fungicides.* A portion of the β-*tub* gene from 17 *B. cinerea* isolates was amplified using primers 155 (5′-CAACCTTCAAAATGCGT GAG-3′) and 1174 (5′-AGATGGGTTGCTGAGCTTCA-3′) (Fekete et al. 2012; Fournier et al. 2005). PCR was performed as described for the *cytb* gene, except that 55°C was used for annealing. PCR products were visualized under UV-light in a 0.75% agarose gel in 1× Tris-acetate-EDTA buffer after ethidium bromide staining. For some isolates, a two-band profile was observed and extracted using the QIAquick Gel Extraction Kit (Qiagen), following the manufacturer's protocol. The β-*tub* gene PCR and gel extraction products were purified, sequenced, and analyzed as described above.

Results

Fungicide sensitivity in mycelial growth assays. EC_{50} values of 67 isolates (67.7% of the total number of isolates) were higher than 71.9 µg/ml for azoxystrobin, and these isolates were considered resistant. There was no inhibition in growth of these isolates with azoxystrobin at 71.9 µg/ml. Among isolates from organic fields, 31.4% showed resistance to the fungicide and 68.6% had EC_{50} values lower than 4.5 µg/ml and were considered sensitive (Fig. 1A). In conventional fields, 87.5% of the isolates were considered resistant to azoxystrobin (Fig. 1A).

EC_{50} values of 57 isolates (57.6% of the total number of isolates collected) for iprodione were equal to or higher than 1.2 µg/ml, and these were considered resistant. Isolates from organic fields had EC_{50} of 0.38 to 5.96 µg/ml (Fig. 1B); 77.1% of them had mean values lower than 1.2 µg/ml and were considered sensitive (Fig. 1B). Mean EC_{50} values for isolates from conventional fields varied from 0.33 to 5.70 µg/ml, and 76.6% of these isolates were considered iprodione resistant (Fig. 1B).

EC_{50} values of 20 isolates (20.2% of the total number of isolates) for pyrimethanil were equal to or higher than 5 µg/ml, and these were considered resistant. Isolates from conventional fields had EC_{50} of 0.56 to 33.4 µg/ml (Fig. 1C); 76.6% of them had mean values lower than 5 µg/ml and were considered sensitive (Fig. 1C). In organic production fields, EC_{50} values ranged from 0.57 to 10.9 µg/ml and 85.7% of the isolates were pyrimethanil sensitive (Fig. 1C). Meanwhile, 12.5% of the isolates collected from conventional areas had EC_{50} values higher than 10 µg/ml, whereas only one isolate from an organic field presented such higher values.

EC_{50} values of 77 isolates (77.8% of the total number of isolates collected) for thiophanate-methyl were higher than 688 µg/ml, and these were considered resistant. Growth of these isolates was not inhibited on PDA amended with thiophanate-methyl at 688 µg/ml. Among isolates from organic fields, 51.4% showed resistance to the fungicide and 48.6% had EC_{50} values lower than 7.5 µg/ml and were considered sensitive (Fig. 1E). Among isolates from conventional fields, 7.8% were considered to be thiophanate-methyl sensitive, with EC_{50} values lower than 3 µg/ml (Fig. 1E). EC_{50} of sensitive isolates collected in organic and conventional fields varied from 0.4 to 7.5 µg/ml.

The overall frequency of *B. cinerea* isolates sensitive to the four tested fungicides was 14.1%, whereas the frequencies of isolates resistant to one (FR1), two (MFR2), three (MFR3), and four (MFR4) chemical classes were 17.2, 13.1, 41.4, and 14.1%, respectively. Among the isolates collected at organic fields, 27.8% were considered sensitive to the four tested fungicides (Fig. 2), whereas 36.1, 22.2, 11.1 and 2.8% were included in the FR1, MFR2, MFR3, and MFR4 groups, respectively. Among the FR1 isolates from organic fields, 5.6, 2.8, 5.6, and 22.2% were resistant to azoxystrobin, iprodione, pyrimethanil, and thiophanate-methyl, respectively (Fig. 2). In the MFR2 phenotype, 13.9% were resistant to both azoxystrobin and thiophanate-methyl, 5.6% were resistant to iprodione and thiophanate-methyl, and 2.8% were resistant to pyrimethanil and thiophanate-methyl (Fig. 2). Among the MFR3 isolates, 5.6% were resistant to azoxystrobin, iprodione, and pyrimethanil and 5.6% were resistant to azoxystrobin, iprodione, and thiophanate-methyl (Fig. 2). Among the isolates collected at conventional fields, 6.3% were considered sensitive to the four tested fungicides (Fig. 2), whereas 6.3, 7.9, 58.7, and 20.6% were included in the FR1, MFR2, MFR3, and MFR4 groups, respectively. In the FR1 phenotype, 1.6 and 4.8% were resistant to azoxystrobin and thiophanate-methyl, respectively (Fig. 2). Among the MFR2 phenotype, 6.3% were resistant to azoxystrobin and thiophanate-methyl and 1.6% were resistant to

iprodione and thiophanate-methyl (Fig. 2). Among the MFR3 isolates, 55.6% were resistant to azoxystrobin, iprodione, and thiophanate-methyl and 3.2% were resistant to azoxystrobin, pyrimethanil and thiophanate-methyl (Fig. 2).

Fungicide sensitivity in spore germination assays. Sensitivity to azoxystrobin was also tested in a spore germination assay for 15 isolates. Spore germination of nine *B. cinerea* isolates was not inhibited by azoxystrobin at concentrations up to 100 μg/ml. The same nine isolates were not inhibited in the mycelial growth assay by azoxystrobin at 71.9 μg/ml. EC_{50} values of the other six isolates tested ranged from 0.25 to 0.91 μg/ml for mycelial growth and from 0.70 to 1.39 μg/ml for spore germination.

Fungicide sensitivity in fruit assays. Field rates of azoxystrobin, iprodione, pyrimethanil, or thiophanate-methyl failed to control BFR on fruit inoculated with isolates resistant to the four fungicides (Table 1). Disease incidence and severity on fruit treated with azoxystrobin and inoculated with sensitive isolates ranged from 61.1 to 91.7 and 50.8 to 86.1%, respectively, whereas fruit inoculated with resistant isolates showed incidence and severity varying from 76.7 to 98.2 and 70.4 to 99.2%, respectively. Incidence and severity of azoxystrobin-treated fruit inoculated with sensitive and resistant isolates were not different from the water-sprayed control. Disease

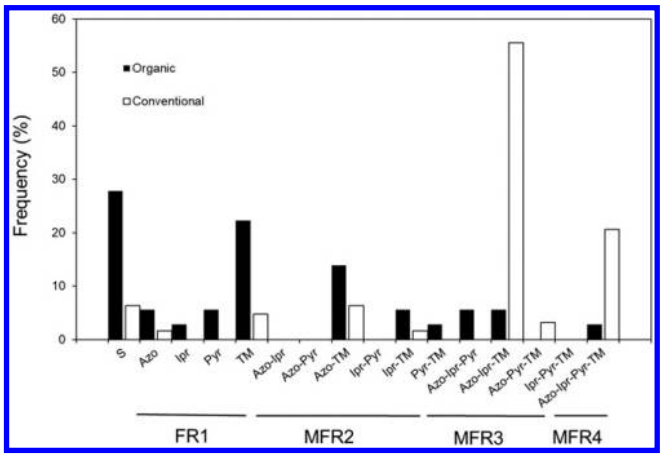

Fig. 2. Overall frequency of fungicide resistance phenotype observed in *Botrytis cinerea* isolates across strawberry organic (black bars) and conventional (white bars) fields to four different fungicides. Azo, Ipr, Pyr, and TM indicate azoxystrobin, iprodione, pyrimethanil, and thiophanate-methyl, respectively. S indicates sensitive isolates, whereas FR1, MFR2, MFR3, and MFR4 indicate isolates that are resistant to one, two, three, and four fungicides, respectively.

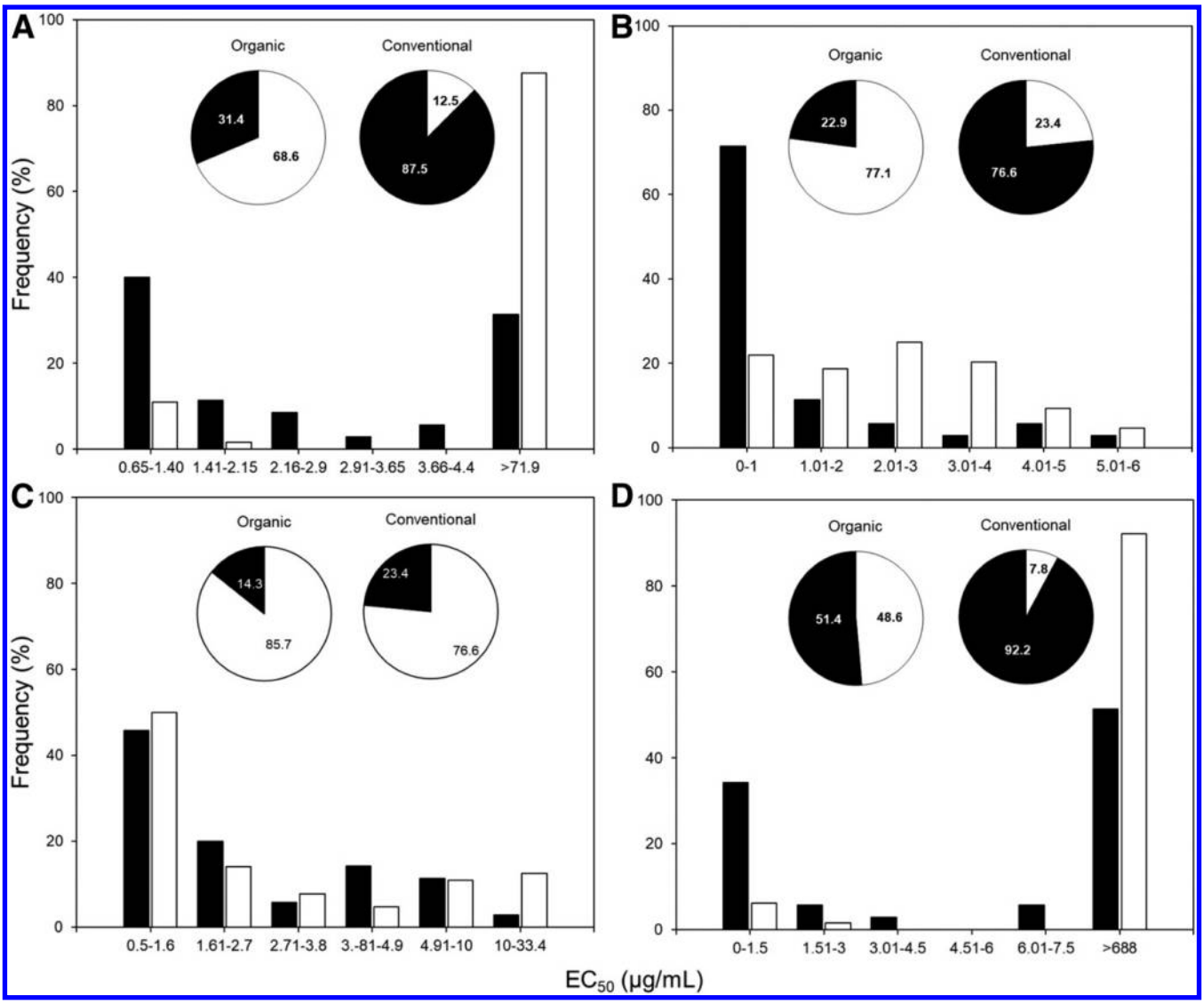

Fig. 1. Sensitivity of isolates of *Botrytis cinerea* to **A,** azoxystrobin; **B,** iprodione; **C,** pyrimethanil; and **D,** thiophanate-methyl. Pie graphs show the frequency of sensitive (white) and resistant (black) isolates collected in organic (n = 35) and conventional (n = 64) strawberry fields. Bar graphs show the frequency of isolates from organic (black bars) and conventional fields (white bars) within specified ranges of effective concentration (in micrograms per milliliter) of fungicide that inhibited mycelial growth of *B. cinerea* isolates by 50% (EC_{50}). Isolates were considered resistant to azoxystrobin, iprodione, pyrimethanil, or thiophanate-methyl if their EC_{50} values were higher than 71.9, 1.2, 5.0, or 688 μg/ml, respectively, in mycelial growth assays.

incidence of iprodione-treated fruit inoculated with resistant isolates ranged from 60.2 to 83.2%. Meanwhile, disease severity ranged from 0.0 to 0.4% for the sensitive isolates and from 20.5 to 73.9% for the fruit inoculated with resistant isolates. BFR incidence and severity of pyrimethanil-treated fruit inoculated with sensitive isolates varied from 14.5 to 53.8 and 4.4 to 29.2%, respectively, whereas, for the resistant isolate, they were 86.1 and 85.9%, respectively. Disease incidence and severity on fruit treated with thiophanate-methyl and inoculated with sensitive isolates ranged from 25.8 to 56.1 and 12.4 to 49.2%, respectively, whereas those for resistant isolates varied from 83.3 to 94.4% and 80.6 to 97.7%, respectively. BFR incidence and severity were usually higher on sprayed fruit inoculated with resistant isolates (Table 1).

Characterization of mutations linked to resistance to QoI, DC, and MBC fungicides. *Analysis of* cytb *gene sequences linked to resistance to QoI fungicides.* The partial *cytb* gene of *B. cinerea* isolates was amplified and fragments of 560 or 1,500 bp were visualized after gel electrophoresis. After restriction digestion of PCR products, the mutation at codon 143 was identified by the presence of two bands of 318 and 242 bp in length, whereas the presence of the fragments 560 and 1,500 bp in length indicated absence of the mutation. Isolates showing a one-band profile after restriction digestion of their PCR products had EC_{50} values for azoxystrobin ranging from 0.66 to 4.34 μg/ml, and isolates presenting a two-band profile after digestion of the PCR products showed an EC_{50} higher than 71.9 μg/ml.

Sequences of PCR products of 22 isolates were compared with two sequences of *Botryotinia fuckeliana* from the GenBank. The sequence with accession number AB262969.1 did not have the mutation at codon 143, and the sequence with accession number AB428335.1 contained an intron between codons 143 and 144 (Bcbi-143/144). Three *cytb* gene profiles were observed among our *Botrytis cinerea* isolates: (i) isolates without the Bcbi-143/144 intron and without the point mutation at position 143 were azoxystrobin sensitive, (ii) isolates without the Bcbi-143/144 intron and with the point mutation G143A were azoxystrobin resistant, and (iii) isolates with the Bcbi-143/144 intron and without the point mutation at 143 were azoxystrobin sensitive (isolates presenting the fragment 1,500 bp in length) (Supplementary Fig. S1). All azoxystrobin-resistant isolates had the mutation G143A and no mutation was observed in any of the sensitive isolates. No mutation at position 137 was observed in any of the sequenced isolates.

Analysis of bos1 *gene sequences linked to resistance to DC fungicides.* Alignment of the partial nucleotide sequences of *bos1* gene from 14 *B. cinerea* isolates exhibiting different sensitivity phenotypes revealed either the absence or presence of mutations at codons 365, 369, and 373 (Table 2). No mutation was observed in any of the iprodione-sensitive isolates. At position 365, isoleucine (I) was replaced by serine (S) in three isolates, or by asparagine (N) in two isolates. In four isolates, glutamine (Q) was replaced by proline (P) and N was replaced by S at codons 369 and 373, respectively (Table 2). Sequences were compared with a sensitive

Table 1. Efficacy of azoxystrobin, iprodione, and thiophanate-methyl in controlling Botrytis fruit rot of strawberry fruit inoculated with *Botrytis cinerea* isolates with different levels of sensitivity to the tested fungicides

Fungicide, isolate[w]	EC_{50} (μg/ml)[x]	Type[y]	Mutations in target genes[z]		Botrytis fruit rot (%)[v]	
					Incidence	Severity
Azoxystrobin						
13-01	>71.9	R	*cytb*	G143A	76.7 ab	70.4 a
13-29	0.99	S	*cytb*	–	91.7 a	86.1 ab
13-34	0.97	S	*cytb*	–	63.9 b	57.7 b
14-16	>71.9	R	*cytb*	G143A	98.2 a	99.2 a
14-41	3.85	S	*cytb*	–	61.1 b	50.8 b
15-03	>71.9	R	*cytb*	G143A	97.2 a	84.0 ab
Iprodione						
13-01	3.07	R	*bos1*	I365S	83.2 a	73.9 a
13-29	0.76	S	*bos1*	–	0.0* c	0.0* c
13-34	0.39	S	*bos1*	–	0.0* c	0.0* c
14-16	1.51	R	*bos1*	I365N	60.2* b	20.5* b
14-41	0.81	S	*bos1*	–	0.0* c	0.4* c
15-03	4.40	R	*bos1*	I365S	77.4* ab	62.7* a
Pyrimethanil						
13-01	0.88	S	N/A	N/A	32.8* b	29.2* b
13-29	2.68	S	N/A	N/A	14.5* b	4.4* b
13-34	2.80	S	N/A	N/A	53.8 ab	17.5* b
14-16	33.49	R	N/A	N/A	86.1 a	85.9 a
14-41	4.04	S	N/A	N/A	25.6* b	5.8* b
15-03	0.95	S	N/A	N/A	37.4* b	11.4* b
Thiophanate-methyl						
13-01	>688	R	β-*tub*	E198A	83.3 a	80.6 a
13-29	1.28	S	β-*tub*	–	25.8* c	12.4* c
13-34	6.34	S	β-*tub*	–	50.0 bc	49.2 b
14-16	>688	R	β-*tub*	E198A	94.4 a	97.7 a
14-41	0.51	S	β-*tub*	–	56.1 b	40.4* b
15-03	>688	R	β-*tub*	E198A	90.8 a	92.7 a

[v] Botrytis fruit rot incidence and severity on fungicide-sprayed fruit were estimated and compared with the water-sprayed control. Data are the means of three replications in each of two experiments. Values in columns followed by the same letters for each fungicide are not significantly different at $P \leq 0.05$ as determined by analysis of variance and least significant difference tests. Means followed by (*) are different from the control as determined by analysis of variance at $P \leq 0.05$.

[w] Fruit were sprayed preventively with azoxystrobin, iprodione, pyrimethanil, and thiophanate-methyl at 80, 750, 600, and 490 μg/ml, respectively.

[x] Effective concentrations that inhibited mycelial growth of *B. cinerea* isolates by 50% (EC_{50}) were determined using the spiral gradient dilution method.

[y] S and R indicate sensitive and resistant phenotypes of *B. cinerea* isolates, respectively, based on EC_{50} values (>71.9, >1.2, >5.0, and >688 μg/ml for azoxystrobin, iprodione, pyrimethanil, and thiophanate-methyl, respectively) and molecular analysis of target genes.

[z] Point mutations identified at specific codons in the cytochrome *b* (*cytb*), *bos1*, and β-tubulin (β-*tub*) genes of *B. cinerea* isolates; – indicates that mutations were absent in sensitive isolates. N/A = not available and indicates that the molecular mechanisms of resistance to aniline-pyrimidines has not yet been determined.

phenotype sequence of *B. cinerea* from GenBank (accession number JX192631.1).

Analysis of β-tub *gene sequences linked with resistance to MBC fungicides.* Nucleotide sequencing analysis of a portion of the β-*tub* gene of 17 *B. cinerea* isolates revealed that, at position 198, glutamic acid (E) was replaced by alanine (A) in thiophanate-methyl-resistant isolates but not in sensitive isolates. No mutations at positions 167 and 200 were revealed in any of the sequenced isolates. Sequences were compared with a sensitive phenotype sequence of *B. cinerea* from GenBank (accession number Z69263.2).

Discussion

Conventional strawberry growers frequently use azoxystrobin, iprodione, pyrimethanil, and thiophanate-methyl to control BFR in Brazil. This study provides information on the occurrence, frequency distribution, and molecular characterization of resistance to single-site fungicides in *B. cinerea* isolates collected in conventional and organic Brazilian strawberry fields. Fungicide resistance in *B. cinerea* isolates was found in almost every conventional commercial field, and 20.6% of the isolates were resistant to all four tested fungicides (MFR4). However, at lower frequencies (2.8%), isolates resistant to all the active ingredients were also recovered from organic strawberry fields.

Similar EC_{50} values of isolates sensitive to azoxystrobin have been reported for *B. cinerea* isolates with no previous exposure to azoxystrobin from vegetables in China, where EC_{50} values ranged from 0.04 to 5.25 μg/ml (Zhang et al. 2011). Sensitive isolates had lower EC_{50} values for spore germination than for mycelial growth, which was also observed in other studies for *B. cinerea* isolates from different hosts (Chatzidimopoulos et al. 2013; Zhang et al. 2011). High resistance frequencies to QoI fungicides in strawberry fields were also reported in the United States, Europe, and Japan (Amiri et al. 2013; Fernández-Ortuño et al. 2016; Ishii et al. 2009).

Only azoxystrobin-resistant isolates had the G143A mutation in the *cytb* gene. This point mutation has been linked to the expression of high or complete resistance, leading to failure of field control of the disease (Banno et al. 2009). It has been extensively documented in resistant isolates from several fungal plant pathogens worldwide, including *B. cinerea* from strawberry and other small fruit (Amiri et al. 2013; Ishii et al. 2009; Leroux et al. 2010). *B. cinerea* isolates possessing the intron Bcbi-143/144 between codons 143 and 144 of the *cytb* gene did not have the G143A mutation, whereas resistant isolates did not have the intron, as already reported by others (Banno et al. 2009; Leroux et al. 2010). It has been suggested that the mutation at codon 143 prevents self-splicing of the 143/144 intron, which may lead to cytochrome deficiency and death of the organism (Banno et al. 2009).

Table 2. Characterization of the partial *bos1* gene of *Botrytis cinerea* isolates collected in organic and conventional strawberry fields in Brazil and EC_{50} mean values for iprodione

Isolates	EC_{50} (μg/ml)[y]	Phenotype[z]	Amino acid at codons of *bos1* gene[x]		
			365	369	373
13-15	0.38	S	I	Q	N
13-34	0.39	S	I	Q	N
14-42	0.52	S	I	Q	N
13-29	0.76	S	I	Q	N
14-41	0.81	S	I	Q	N
14-16	1.51	R	**N**	Q	N
14-38	2.12	R	**N**	Q	N
13-01	3.07	R	**S**	Q	N
15-03	4.40	R	**S**	Q	N
13-28	4.49	R	**S**	Q	N
15-01	2.25	R	I	**P**	**S**
14-02	3.37	R	I	**P**	**S**
14-05	3.81	R	I	**P**	**S**
14-14	3.94	R	I	**P**	**S**

[x] Amino acids serine (S), isoleucine (I), glutamine (Q), proline (P), and asparagine (N) coded at positions 365, 369, and 373 of *bos1* gene. Bold and underline letters represent the amino acid replacements in the resistant phenotype isolates.

[y] Average effective concentration of iprodione that inhibited mycelial growth by 50% (EC_{50}) using the spiral gradient dilution method.

[z] S and R = sensitive and resistant phenotypes, respectively, of *B. cinerea* isolates.

Field rates of commercial formulations of azoxystrobin failed to control BFR on detached fruit inoculated with *B. cinerea* isolates. Although disease incidence and severity in fruit inoculated with sensitive isolates were statistically different from the values observed for fruit inoculated with resistant isolates, they did not differ from the values found in the water-sprayed control. Many plant pathogens, including *B. cinerea*, develop an alternative respiration pathway that allows electron flux to bypass the blockage of the cytochrome pathway caused by the QoI, avoiding the toxic effects of the fungicide (Leroux et al. 2002; Tamura et al. 1999). Under field conditions, plant components may have an important role in the control of BFR, because naturally occurring substances (i.e., flavonoids) can inhibit the activity of the alternative oxidase pathway of *B. cinerea* (Tamura et al. 1999). However, field efficiency of QoI could be compromised and *B. cinerea* may survive after fungicide treatments, because strawberry fruit have high levels of quercetin, a flavonoid that showed no inhibition of the function of the alternative oxidase in *B. cinerea* (Häkkinen et al. 1999; Tamura et al. 1999). Moreover, studies have shown that azoxystrobin has a fungistatic rather than a fungicidal action (Inoue et al. 2012) and could explain why the sensitive isolates were not completely controlled. In fact, azoxystrobin is registered as a BFR suppressor only for strawberry in the United States, and is not registered for BFR in Brazil. Additionally, field rates used in Brazil (80 μg/ml) are lower than in Florida (117 to 302 μg/ml) (AGROFIT 2017; Whitaker et al. 2017). However, because azoxystrobin is being used for control of other strawberry diseases, the fungicide may impose selection pressure on *B. cinerea* QoI-resistant strains.

In our studies, isolates of *B. cinerea* with EC_{50} values higher than 1.2 μg/ml were considered iprodione resistant, and resistance was conferred by point mutations in the *bos1* gene at codons 365, 369, or 373. Isolates with EC_{50} for mycelial growth higher than 1.1 μg/ml collected by others had the same substitutions in the target gene (Ma et al. 2007). Although our results showed that isolates with mean EC_{50} values as high as 0.81 μg/ml did not have any mutations in the target gene, some authors consider isolates growing at 0.4 μg/ml to be resistant (Banno et al. 2009), whereas others used 25 μg/ml as a discriminatory dose for identifying resistance (Rupp et al. 2017). Low to moderate resistance levels are typically found in isolates collected from fields, whereas high resistance is rarely seen in the field but has been reported for laboratory mutants (Grabke et al. 2014; Leroux et al. 1999). Mutations at codon 165 (I365 N/S) were also reported for low-resistant isolates of *B. cinerea* collected at strawberry fields in the United States and in Spain (Fernández-Ortuño et al. 2016; Grabke et al. 2014). Moreover, isolates with the I365N mutation and lower EC_{50} values than isolates with the I365S mutation were also observed in other studies (Fernández-Ortuño et al. 2016; Ma et al. 2007). Isolates with EC_{50} values ranging from 2.25 to 3.94 μg/ml had mutations at codons 369 (Q369P) and 373 (N373S), similar to those previously observed in *B. cinerea* isolates with EC_{50} values ranging from 2.48 to 2.68 μg/ml (Ma et al. 2007).

In our studies, BFR symptoms on fruit treated with field rates of commercial formulations of iprodione and inoculated with DC-sensitive isolates were inhibited. Although no mutations in the *bos1* gene was observed in our sensitive isolates, according to other authors, its EC_{50} mean values can characterize it as a low-resistant isolate (Banno et al. 2009).

Isolates of *B. cinerea* from strawberry with EC_{50} values lower than 5 μg/ml were considered to be pyrimethanil sensitive based on previous studies from Amiri et al. (2013). The same study classified *B. cinerea* isolates as moderately resistant or highly resistant when EC_{50} mean values were 5 to 10 and >10 μg/ml, respectively. According to this classification, only a few isolates collected at conventional fields in our study could be considered highly resistant to pyrimethanil. The

overall frequency of resistant isolates found in Brazilian strawberry fields is less than half of the approximately 60% resistance frequency reported in Florida strawberry fields (Amiri et al. 2013). It was also reported in China that *B. cinerea* isolates of strawberry resistant to pyrimethanil had EC_{50} values ranging from 9.4 to 57 μg/ml (Yin et al. 2014), similar to our observations. On the other hand, resistance frequencies for *B. cinerea* isolates from vegetable crops and vineyards isolates were calculated based on discriminatory EC_{50} values of 0.1 and 0.5 μg/ml, respectively, for mycelial growth using a minimal medium similar to CzA (Leroux et al. 1999; Myresiotis et al. 2007). Among the isolates resistant to pyrimethanil, 75% were also resistant to iprodione. This could be explained by the fact that these active ingredients are combined in prepackaged mixtures in Brazil (AGROFIT 2017).

Although disease incidence and severity in fruit inoculated with AP-sensitive isolates were statistically lower than for fruit inoculated with the resistant isolate and differed from the values observed for the water-sprayed control, field rates of commercial formulations of pyrimethanil did not completely control BFR, as observed by Amiri et al. (2013). However, field rates of pyrimethanil registered in Brazil and used in our study are lower than those in Florida (840 μg/ml) (Amiri et al. 2013).

The intensive use of MBC fungicides since their introduction in the 1960s in Brazil, the high risk of *B. cinerea* for resistance development, and the absence of fitness cost associated with MBC resistance can explain the high incidence of *B. cinerea* isolates resistant to thiophanate-methyl (FRAC 2013; Leroux et al. 2002; Raposo et al. 1996). Similar high frequencies of resistance to MBC fungicides have already been reported (Myresiotis et al. 2007).

Isolates of *B. cinerea* resistant to thiophanate-methyl with EC_{50} values as high as in our studies have already been reported (Mercier et al. 2010; Weber and Hahn 2011). A mutation at codon 198 (E198A) in the β-*tub* gene was observed in all of the sequenced resistant isolates but not in the sensitive isolates. This mutation is usually found in highly resistant isolates of *B. cinerea*, and increased fungicide concentration cannot control BFR (Chatzidimopoulos et al. 2014; Fernández-Ortuño and Schnabel 2012; Leroux et al. 2002). A mutation at position 200 (F200Y) is frequently related to moderately resistant strains (Banno et al. 2008; Chatzidimopoulos et al. 2014; Leroux et al. 2002) but none of our isolates had this mutation.

Detached fruit treated with thiophanate-methyl and inoculated with resistant and sensitive isolates of *B. cinerea* developed BFR symptoms. However, BFR incidence and severity were statistically lower in fruit inoculated with MBC-sensitive isolates. Similar results were observed for MBC-resistant isolates collected from strawberry fields in the southern United States (Fernández-Ortuño and Schnabel 2012).

Isolates with lower EC_{50} values to all the tested fungicides were more commonly obtained from organic farms as compared with conventional areas. Moreover, MFR2 and MFR3 phenotypes were more frequently recovered at conventional fields, whereas resistant isolates from organic areas were usually included in the FR1 group. However, considering that site-specific fungicides are not permitted in organic strawberry production, the presence of resistant *B. cinerea* populations in organic farms is intriguing, despite the lower frequencies observed. This fact could be explained by the risk of cross contamination and gene flow between fields due to the proximity of conventional to organic fields, because strawberry production takes place in concentrated regions within the southeast states of Brazil. Therefore, the spray programs in conventional fields could affect the occurrence of resistance in organic fields. Another reason might be the introduction of new populations of *B. cinerea* every year via transplants from different nurseries, because strawberry are grown as an annual crop in Brazil. Additionally, *B. cinerea* populations resistant to single-site fungicides were recovered from strawberry nursery plants in the United States and Germany prior to transplanting to production fields (Oliveira et al. 2017; Rupp et al. 2017). This can explain the introduction of *B. cinerea*-resistant isolates to organic fields, considering the absence of organic production of strawberry transplants in Brazil. Moreover, many conventional and organic growers acquire strawberry transplants from Brazilian strawberry nurseries, due to low prices; however, they do not achieve the demanded standard for nursery certification (de Oliveira and Scivittaro 2006), which can also lead to the overall emergence of fungicide resistance observed in our studies.

In Brazil, there is a limited number of active ingredients registered for strawberry and high frequencies of resistant isolates of *B. cinerea* were observed for azoxystrobin, iprodione, and thiophanate-methyl in our studies. *B. cinerea* isolates were highly resistant to azoxystrobin and thiophanate-methyl and the use of higher fungicide rates in the field is not feasible and will not control these resistant isolates (Deising et al. 2008). However, despite the emergence of resistant isolates, Brazilian strawberry growers use higher rates of iprodione and achieve satisfactory control of BFR.

The occurrence of *B. cinerea* isolates resistant to multiple classes of fungicides worldwide requires modification in BFR management, including new control approaches. Considering the high frequencies of isolates with multiple resistance to the most commonly used fungicides, as well the restricted number of active ingredients available with different modes of action registered for strawberry, future perspectives on successful management of diseases in Brazil are uncertain. Therefore, disease control, in general, on strawberry should be achieved by integrating chemical and cultural control methods (i.e., with reduced fungicide application programs accompanied by monitoring of the resistance, use of less susceptible cultivars, control of irrigation, use of tunnels, and acquisition of healthy propagation material, among others). Constant inspections and monitoring for disease symptoms and fungicide resistance development in nurseries as well as their certification should be crucial for disease management of strawberry production systems.

This study reinforces the importance of continuous research and monitoring of risks associated with fungicide resistance occurrence, the urgency for the implementation of resistance management programs, and the need for an integrated approach between strawberry nurseries and production fields for BFR management.

Acknowledgments

We thank H. Costa for providing some of the isolates used in this study and B. Momesso, S. A. Lourenço, and T. E. Seijo for technical assistance.

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