Baseline Sensitivity of *Botrytis cinerea* Isolates from Strawberry to Isfetamid Compared to other SDHIs

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Abstract

Succinate dehydrogenase inhibitors (SDHIs) are the fungicides most commonly used to control *Botrytis* fruit rot on commercial strawberry in Florida. The medium-to-high risk of selection of resistance in the causal agent *Botrytis cinerea* is a threat to the efficacy of this fungicide group. In this study, we characterized the sensitivity of *B. cinerea* to the SDHI isfetamid, evaluated the SdhB gene mutation associated with resistance, and monitored resistance frequencies to five SDHI fungicides for two consecutive seasons. EC₅₀ values of 70 isolates were obtained using the spiral gradient dilution (SGD) method and averaged 0.098 µg/ml of isfetamid. EC₅₀ averages of 3.04 and >500.00 µg/ml were obtained for isolates with the N230I and P225F mutations indicating moderate and high resistance to isfetamid, respectively. A total of 565 *B. cinerea* isolates collected during 2015–2016 and 2016–2017 seasons from strawberry nurseries and Florida production fields were evaluated using conidial germination

assays. Results for the first season showed resistance frequencies of 95, 33, 21, 25, and 0% to boscalid, penthiopyrad, fluopyram, benzovindiflupyr, and isfetamid, respectively. The respective resistance frequencies for the following season were 91, 95, 44, 27, and 1.3%. Only three isolates were found to be moderately resistant to isfetamid during the second season, and the mutation N230I was identified after sequence analysis. These isolates were confirmed to be resistant to isfetamid in fruit assays with disease incidence of 55.6 to 77.0%; however, the conidial production of the isolates was inhibited by an average of 83.9%. In general, isfetamid efficacy was higher than the other evaluated SDHIs, but a slight increase in resistance frequencies was observed in our study.

Keywords: BFR, fungi, fruit, small fruits, disease management, fungicide resistance

Botrytis cinerea is the causal agent of *Botrytis* fruit rot (BFR), one of the most significant diseases of strawberry in the United States and worldwide (Mertely et al. 2018). During the strawberry season in Florida, temperatures ranging from 15 to 25°C with leaf wetness greater than 13 consecutive hours that are optimal for BFR development are common (Bulger et al. 1987). The infection starts as small, hard necrotic spots that can occur on fruit stem and flower parts like petals. Under favorable conditions, the rotting lesions can be entirely covered with masses of conidia that can rapidly spread through the field causing significant yield losses (Williamson et al. 2007). BFR incidence higher than 50% has been reported on fruit harvested from nontreated plots (Cordova et al. 2015), indicating the importance of an effective disease management program.

BFR management in Florida's commercial fields relies strongly on the use of fungicides. Traditional calendar-based programs consist of protective weekly applications of multisite fungicides such as captan or thiram throughout the season starting after plant establishment with applications of single-site fungicide applications during periods of peak bloom if weather conditions favor BFR development (Legard et al. 2005). This management approach can result in more than 20 applications in a season, many of which may be unnecessary. Cordova et al. (2017a, b, 2018) showed that strawberry growers following the Strawberry Advisory System (StAS) may reduce the number of fungicide applications to manage BFR by approximately 50% with no harm to yield or disease suppression. The high number of fungicide applications, besides increasing costs, also increase the selection pressure for resistant

B. cinerea populations. Furthermore, this selection might occur at the nursery level, with the use of nontarget BFR sprays to manage other diseases (Oliveira et al. 2017).

B. cinerea is considered a high-risk pathogen for the development of fungicide resistance because of its polycyclic life cycle, profuse sporulation, broad host range, and high genetic variability (Fernández-Ortuño et al. 2012; Veloukas et al. 2014). This variability makes the pathogen complex and resistant to a vast number of registered fungicides within the succinate-dehydrogenase inhibitor (SDHI), quinone-oxidase inhibitor (QoI), anilinopyrimidine (AP), and hydroxanilide (Hyd) fungicide groups (Amiri and Peres 2014; Kumari et al. 2014). In past years, the SDHI fungicides have become an important and effective tool for BFR control (Hu et al. 2016). The SDHIs are the group incorporating new broad-spectrum compounds in the market most rapidly. They have the specific function of inhibiting the pathogen mitochondrial respiration by targeting the ubiquinone-binding pocket in the mitochondrial respiratory complex II or SDH, compromising SDH-A (flavoprotein), SDH-B (iron-sulfur protein), and SDH-C and D (two membrane-anchored proteins) (Hu et al. 2016; Sierotzki and Scalliet 2013).

Regardless of the SDHIs high fungicide efficacy, these fungicides are classified as having a medium-to-high risk of resistance (Amiri et al. 2014; FRAC 2017). *B. cinerea* resistance to SDHI fungicides has been associated with genetic modifications in the SDH subunits B, C, and D. The most frequent and common mutations are in the SDH-B, H272R, and H272Y, the result of the histidine replacement at codon 272 by arginine and tyrosine, respectively. A less common mutation of the same codon is H272L, replacing histidine for leucine. Other mutations described in the subunit B are N230I, with the replacement of asparagine by isoleucine at codon 230, and P225F, P225L, and P225T, where genetic modification occurs at codon 225 with the substitution of proline by phenylalanine, leucine, and threonine, respectively (Fernández-Ortuño et al. 2012; Veloukas et al. 2014).

The mutations mentioned above are responsible for conferring different levels of resistance or phenotypes to SDHI fungicides on *B. cinerea*. In the case of boscalid, the first SDHI fungicide released in Florida in 2003, and penthiopyrad, released in 2012, moderate to very high resistance can be observed in *B. cinerea* isolates with H272R/Y, N230I, and P225F mutations. Moreover, only N230I

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and P225F have been reported to confer high to very high resistance on fluopyram, one of the newer SDHI fungicides, released in 2015 (Amiri et al. 2014).

Isofetamid is the newest SDHI released for use on strawberry, but resistance has not yet been investigated. Piqueras et al. (2014) demonstrated the high efficacy of isofetamid for control of *B. cinerea* on grape. Isofetamid is also used on berries, lettuce, turfgrass, and rape-seed to control diseases caused by *Botrytis* and *Sclerotia* spp. (Jeanmart et al. 2016).

Amiri et al. (2013, 2014) showed that 80% of the *B. cinerea* isolates from strawberry fields were resistant to boscalid and that there was rapid selection of resistance to penthiopyrad, which indicates the importance of a periodic resistance evaluation of old and newly registered fungicides from the SDHI group. Thus, the objectives of this study were to (i) determine the baseline sensitivity of *B. cinerea* isolates collected from Florida strawberry fields to isofetamid; (ii) identify the mutation(s) within the SdhB subunit conferring resistance to isofetamid; and (iii) monitor the frequency of fungicide resistance among strawberry *B. cinerea* isolates from nurseries and Florida production fields to boscalid, penthiopyrad, fluopyram, benzovindiflupyr, and isofetamid.

Materials and Methods

***B. cinerea* isolate collection.** Seventy *B. cinerea* isolates collected prior to isofetamid registration for strawberry in 2015 were selected to establish the baseline sensitivity for isofetamid (Table 1). Isolates were collected over a period of 8 years from 2001 to 2013 from symptomatic strawberries produced in different locations in Florida and were preserved in glycerol as conidial suspensions at the Gulf Coast Research and Education Center (GCREC) in Wimauma, FL at -80°C . To revive the isolates, a small portion of the suspension was transferred to HA medium (Leroch et al. 2013).

Twelve isolates of *B. cinerea* previously characterized for boscalid sensitivity and their associated mutation in the subunit B of the SDH (Amiri et al. 2014) were characterized for isofetamid sensitivity in this study as described in the section below (Table 2).

For the monitoring of SDHI sensitivity, 565 isolates of *B. cinerea* were collected from symptomatic strawberry fruit and detached leaves from transplants during two consecutive seasons. In the 2015–2016 strawberry season, 141 isolates were recovered from symptomatic fruit produced in five different commercial strawberry fields in Hillsborough County, Florida. During the same season, 188 isolates were obtained from transplants from 12 nurseries: five from Nova Scotia, one from Ontario, and one from Quebec in Canada, three from North Carolina, and two from California in the United States. In the 2016–2017 season, 166 isolates were collected from four commercial fields in Florida and 70 isolates from transplants grown in one strawberry nursery in Nova Scotia, Canada.

Isolates recovered from symptomatic fruit were obtained by vertically cutting the fruit in half to transfer a small piece of infected tissue to HA medium. Isolates from strawberry transplants were recovered following the leaf assay protocol of Oliveira et al. (2017).

Baseline sensitivity of *B. cinerea* isolates to isofetamid and phenotypic characterization. Sensitivity of isolates to isofetamid (Kenja 400SC, SummitAgro, NC) was determined by obtaining EC_{50} values with the spiral gradient dilution (SGD) method previously developed by Förster et al. (2004) and modified by Amiri et al. (2013). HA medium was used instead of 2% malt extract agar for incubation of isolates prior to preparation of conidial suspensions. Using the spiral gradient endpoint (SGE) software (Spiral System, Autoplate 4000 model; Spiral Biotech), three stock concentrations were calculated that would correspond to three potential EC_{50} ranges based on the molecular weight of the fungicide. The three ranges of concentration used were (i) 0.004 to 0.697, (ii) 0.290 to 44.355, and (iii) 5.791 to 887.096 $\mu\text{g/ml}$. Stock concentrations were prepared with sterile deionized water and stored at 4°C . EC_{50} values for the baseline sensitivity of isofetamid were obtained using the two lower stock concentrations and the assay was performed twice.

For the phenotypic characterization to isofetamid of isolates harboring the P225F mutation, the experiment was performed twice

using the highest concentration range; whereas for sensitive isolates and isolates harboring H272R/Y and N230I mutations, the experiment was performed four times using the remaining two concentration ranges. The EC_{50} values were used to classify the isolates in three different sensitivity phenotypes: sensitive (S), moderately resistant (MR), and highly resistant (HR) with EC_{50} values below 1 $\mu\text{g/ml}$, between 1 and 5 $\mu\text{g/ml}$, and greater than 5 $\mu\text{g/ml}$, respectively.

Sensitivity of *B. cinerea* isolates to SDHI fungicides. Isolates collected during the two strawberry seasons were analyzed for SDHI

Table 1. *Botrytis cinerea* isolates used to determine isofetamid baseline sensitivity

Year ^y	Origin	Isolates ^z
2001	-	01-36, 01-37
2003	Monticello	03-37
2005	Dover	05-25, 05-28, 05-29, 05-30
2008	Balm	08-96, 08-98, 08-100
2010	Balm	10-31, 10-39, 10-40
2010	Lake Wales	10-36, 10-38
2010	Dover	10-41
2011	Dover	11-07, 11-08, 11-09, 11-10
2011	Plant City	11-20, 11-106, 11-109, 11-111, 11-350
2011	-	11-45, 11-48, 11-55, 11-57
2012	Plant City	12-20, 12-50, 12-51, 12-248, 12-249, 12-345, 12-346, 12-347, 12-348
2012	Dover	12-84, 12-85, 12-86, 12-93, 12-95
2012	Balm	12-176, 12-178
2012	Floral City	12-320, 12-321, 12-322
2012	Wimauma	12-384, 12-385, 12-386
2012	Riverview	12-428, 12-429, 12-430
2013	Balm	13-03, 13-15, 13-16, 13-17
2013	Plant City	13-152, 13-153, 13-154, 13-155, 13-184, 13-185, 13-186, 13-188
2013	Floral City	13-390, 13-391, 13-392, 13-417

^y Year of collection. All isolates were collected from different cities in Florida prior to isofetamid registration.

^z Isolates from culture collection of the Gulf Coast Research and Education Center, University of Florida, Wimauma, FL.

Table 2. Phenotypic characterization of succinate dehydrogenase subunit B (SdhB) mutations to isofetamid

Genotype ^z	Isolate	Boscalid ^x	Isofetamid ^y	
		Phenotype	EC_{50}	Phenotype
WT	11-45	HS	0.18	S
	12-221	HS	0.30	S
H272R	12-241	S	0.22	S
	11-67	MR	0.30	S
	10-35	HR	0.19	S
	12-332	HR	0.26	S
H272Y	12-65	VHR	0.01	S
N230I	12-450	VHR	1.46	MR
	12-255	HR	4.79	MR
	17-94	-	1.71	MR
	17-111	-	3.52	MR
	17-222	-	3.70	MR
P225F	11-62	VHR	>500.00	HR
	12-355	VHR	>500.00	HR
	12-374	VHR	>500.00	HR

^x Phenotype of the SdhB mutations conferring different levels of resistance on *Botrytis cinerea* isolates to boscalid according to Amiri et al. (2014). HS (highly sensitive), S (sensitive), MR (moderately resistant), HR (highly resistant), and VHR (very highly resistant). Isolates 12-241 and 12-332 showed in this study a substitution of histidine by arginine at codon 272, different from previously reported by Amiri et al. (2014).

^y Phenotypes for isofetamid were determined based on EC_{50} values obtained in this study. S = sensitive ($<1 \mu\text{g/ml}$), MR = moderately resistant (1 to 5 $\mu\text{g/ml}$), and HR = highly resistant ($>5 \mu\text{g/ml}$).

^z Mutations in the subunit B of the SDH complex. WT = wild type.

sensitivity based on a conidial germination assay. After incubation of the isolates on HA for 6 to 7 days, individual conidial suspensions of 10^6 spores/ml were prepared using a hemacytometer. Petri dishes (15 cm diameter) containing yeast bacto-acetate agar (YBA) (10 g/liter bacto-peptone, 20 g/liter sodium acetate, 10 g/liter yeast extract, 15 g/liter agar) were divided into 30 rectangles measuring 1.5×2.0 cm, allowing the testing of 30 isolates per plate. Using a graduated 10- μ l pipette, two 7- μ l drops of conidial suspension were placed diagonally in each space.

YBA medium was amended with discriminatory doses of 1 or 5 μ g/ml of penthiopyrad (Fontelis, DuPont, Wilmington, DE), 2 or 5 μ g/ml of fluopyram (Luna Privilege, Bayer CropScience, Research Triangle Park, NC), and 2 or 5 μ g/ml of boscalid (Endura, BASF, Research Triangle Park, NC) each. These doses had been previously determined by Amiri et al. (2013) to differentiate isolate sensitivity to each respective fungicide, and the YBA medium was chosen based on Weber and Hahn (2011). Doses for benzovindiflupyr (provided from Syngenta Crop Protection for experimental use) and isofetamid (Kenja 400SC, SummitAgro, NC) were 1 or 5 μ g/ml, each. Nonamended YBA medium was used as a control. All plates were incubated at 23°C for 18 to 24 h, and evaluation of conidial germination was based on a method developed by Weber and Hahn (2011) to classify isolates as sensitive (S), moderately resistant (MR), or highly resistant (HR). The percentage of resistant isolates for each fungicide and multifungicide resistance among the SDHIs tested were determined considering MR and HR isolates compared with the total number of isolates. The assay was performed twice and the combination of one plate per isolate/fungicide-concentration was used.

Characterization of SdhB mutations in resistant isolates of *B. cinerea*. Twenty-three *B. cinerea* isolates resistant to SDHIs collected in the 2015–2016 and 2016–2017 strawberry seasons were grown on HA medium at 23°C for 5 to 7 days. Mycelium from each isolate was collected and DNA extraction was performed using FastDNA Kit (MP Biomedicals).

The primers BcSdhB-F1 (Amiri et al. 2014) and IpBecEnd2 (Leroux et al. 2010) were used to amplify a fragment (850 bp) of the SdhB. Polymerase chain reaction (PCR) was conducted on 25- μ l volume containing 14.6 μ l of molecular water, 5 $\times$ buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPS, 0.2 μ M each primer (10 mM), 2 units Taq polymerase, all from Promega Corp, and 20 ng/ μ l DNA, following the same parameters described by Amiri et al. (2014). PCR products were sequenced at Genewiz in New Jersey, U.S.A. The software

BioEdit version 7.0.5.3 (Hall 1999) was used to assemble sequences and Mega7 version 7.0.2.6 (Kumar et al. 2016) for translation and alignment.

Fruit assays. In vivo evaluations of isofetamid efficacy were conducted on strawberry fruit of ‘Florida Radiance’ with no prior exposure to the fungicide. Fruit with white to pinkish color were harvested from an experimental area at the GCREC, and the methodology described by Amiri et al. (2013) was followed for fruit preparation and inoculation. Prior to inoculation, fruit were sprayed with Kenja, a.i. isofetamid, at 1.2 ml/liter until complete coverage of the fruit was achieved. Three moderately resistant *B. cinerea* isolates harboring the N230I mutation and two sensitive isolates were used for inoculation. Fruit were incubated in growth chambers at 23°C for 6 days, and BFR incidence was determined by the number of symptomatic fruit that were sprayed with isofetamid compared with the control sprayed with water. The experiment consisted of six fruit per isolate with four replications, and the assay was conducted twice. The conidial production was also evaluated on detached fruit following the protocol by Amiri et al. (2014). For this, conidia were collected using one cotton swab per fruit and placed in 1 ml of sterile distilled water, and then counted using a hemacytometer.

Statistical analysis. Repeated experiments for phenotypic characterization and baseline sensitivity were combined after analysis of variance was tested using the generalized linear mixed model, and means were separated using Fisher’s protected least significant difference (LSD). Analysis of variance was performed for data from in vivo evaluations of isofetamid for BFR incidence and conidial production, and means were separated using LSD test. All statistical analyses for this study were conducted using the SAS software (version 9.4; SAS Institute Inc.).

Results

Baseline sensitivity of *B. cinerea* isolates to isofetamid. The EC_{50} values of isolates exposed to isofetamid ranged from 0.007 to 0.515 μ g/ml (Fig. 1) with a mean value of 0.050 μ g/ml. Regardless of the relatively wide EC_{50} range, most of the baseline EC_{50} values (51.4%) were below 0.050 μ g/ml and 31.4% between 0.053 and 0.150 μ g/ml. EC_{50} values that were from 0.155 to 0.490 μ g/ml represented 15.7% of the population, and only 1.4% (1 isolate) had an EC_{50} value higher than 0.500 μ g/ml. The EC_{50} values were over 0.155 μ g/ml for isolates collected from 2005 through 2013.

Phenotypic characterization of SdhB mutations in resistant isolates of *B. cinerea*. Isolates 11-45 and 12-241 that were sensitive to boscalid and had no mutation on the SdhB were also S to isofetamid with EC_{50} values of 0.18 and 0.22 μ g/ml, respectively (Table 2). Isolates with mutations H272R (10-35, 11-67, 12-221, and 12-332) and H272Y (12-65) were also characterized as S to isofetamid according to their corresponding EC_{50} values, 0.19, 0.30, 0.30, 0.26, 0.01 μ g/ml. The isolates 12-450 and 12-255 harboring the mutation N230I with EC_{50} values of 1.46 and 4.79 μ g/ml were characterized as MR. The EC_{50} values for isolates 11-62, 12-355, and 12-374 harboring the P225F mutation were greater than 500 μ g/ml, conferring high resistance to isofetamid. Three additional isolates, 17-94, 17-111, and 17-222, collected during the 2016–2017 season, showed EC_{50} values of 1.71, 3.52, and 3.70 μ g/ml, respectively, and were characterized as MR. These results match the monitoring using the conidial germination assay where these isolates were also characterized as MR.

Sensitivity of *B. cinerea* isolates to SDHI fungicides. During the 2015–2016 season, boscalid resistance frequencies were 97.9% in isolates collected from Florida farms and 93.6% in isolates from nurseries (Fig. 2A). Resistance frequencies of isolates from Florida farms and nurseries were 33.3 and 33.5% for penthiopyrad, and 22.7 and 19.7% for fluopyram, respectively. Different results were observed for benzovindiflupyr sensitivity, in which the frequency of resistant isolates collected from strawberry farms was 31.2% compared with 20.2% for isolates from nurseries. Resistance to isofetamid was not observed in isolates from either location during 2015–2016. In the following season, 2016–2017, resistance frequency from Florida farms and nursery isolates for boscalid was

Fig. 1. Frequency distribution of effective concentration at which mycelial growth was inhibited by 50% (EC_{50}) for 70 *Botrytis cinerea* baseline isolates to isofetamid. Fungicide sensitivity was determined using spiral gradient dilution (SDG) method. Isolates were collected from strawberry fruit from different areas in Florida and had never been exposed to isofetamid. Values on the X-axis indicate the individual isolates grouped in EC_{50} ranges with intervals of 0.05 μ g/ml.

94.6 and 85.7% (Fig. 2B), and for penthiopyrad was 95.8 and 92.9%, respectively. Differences among isolates were observed for fluopyram and benzovindiflupyr depending on their origin. The resistance frequency of isolates from strawberry farms to fluopyram was 56.6%, whereas only 14.3% of the nursery isolates were resistant. In the case of benzovindiflupyr, the respective frequencies were 36.1 and 4.3%. In 2016–2017, resistance to isofetamid was found in only 1.8% of isolates from Florida farms.

Phenotypic characterization of 2015–2016 isolates (Fig. 2A) resulted in a small population of *B. cinerea* being S (4.6%) and MR (1.8%) to boscalid, whereas the remaining 93.6% were HR. Penthiopyrad phenotypic characterization frequencies were 15.8% HR, 17.6% MR, and 66.6% S. Results for fluopyram showed frequencies of 79% S, 1.3% MR, and 9.7% HR. Similar results were obtained for benzovindiflupyr with frequencies of 75.1% S, 17% MR, and 7.9% HR. All 329 isolates were S to isofetamid, indicating the fungicide inhibited *B. cinerea* spore germination of isolates collected during the first evaluated season. Isolates with a high level of resistance (91.5%) were observed for boscalid in the following season, 2016–2017, and only 8.1% were S (Fig. 2B). The MR (56.8%) and HR (38.1%) isolates were observed for penthiopyrad, while S isolates were at 5.1%. Isolates with S phenotype to fluopyram were

55.9% of the population tested during this season, whereas HR isolates were at 35.2%. Isolates considered S, MR, and HR to benzovindiflupyr were 73.3, 20.8, and 5.9%, respectively. Resistance to isofetamid was first observed during the second season, with only 1.3% of the population classified as MR and the remaining 98.7% as S.

Fungicide resistance (FR) for *B. cinerea* isolates to the five SDHIs was evaluated for the 2015–2016 and 2016–2017 seasons combined (Fig. 3). Resistance to one fungicide, boscalid, was observed in 198 isolates representing 35% of the population tested ($n = 565$), and eight isolates corresponding to 1.4% for penthiopyrad. The frequencies of isolates resistant to two fungicides (2FR) was 22.1% for boscalid and penthiopyrad, and 1.2% for boscalid and benzovindiflupyr. Resistance to three fungicides (3FR) always occurred for boscalid, penthiopyrad, and benzovindiflupyr or fluopyram with 5 and 11.2%, respectively. *B. cinerea*-resistant isolates to four (4FR) SDHIs (boscalid, penthiopyrad, benzovindiflupyr, and fluopyram) were 18.9%, whereas 0.5%, three isolates, were resistant to all five fungicides (5FR), including isofetamid.

Characterization of SdhB mutations in resistant isolates of *B. cinerea*. Sequences from the SdhB region were aligned to a *B. cinerea* isolate without mutations (GenBank accession KR866382.1).

Fig. 2. Frequency of fungicide sensitivity of *Botrytis cinerea* isolates collected from strawberry nurseries and Florida fields during 2015–2016 (A) and 2016–2017 (B) strawberry seasons to five succinate dehydrogenase inhibitors (SDHI) fungicides. The fungicide concentrations used were 2 or 5 $\mu\text{g/ml}$ of boscalid (Endura) and fluopyram (Luna Privilege), and 1 and 5 $\mu\text{g/ml}$ of penthiopyrad (Fontelis), benzovindiflupyr (Aprovia, not registered for strawberry), and isofetamid (Kenja) each. Conidial germination data were used to determine frequency of resistance to the fungicides in the evaluated nurseries and Florida fields. S = sensitive, MR = moderately resistant, HR = highly resistant.

The translated amino acid sequence revealed a substitution of asparagine by isoleucine at codon 230 in the three isolates (17-94, 17-111, and 17-222) found to be moderately resistant to isofetamid collected during 2016–2017 (Table 3). Sequences for the 20 arbitrarily selected resistant isolates to other SDHIs from both seasons identified the mutations H272R and N230I in seven and 13 isolates, respectively (Table 3).

Fruit assays. Preventive application of isofetamid effectively controlled S isolates (05-26 and 17-97) but failed to control isolates with N230I mutations (Table 4). In comparison with S isolates, BFR incidence was significantly higher on MR isolates 17-94, 17-111, and 17-222 with 55.6, 77.0, and 72.1%, respectively. A significant difference was also observed in the conidial inhibition between S and MR isolates. Conidial production was completely inhibited on S isolates, whereas on isolates harboring the N230I mutation, the inhibition ranged from 70.7 to 91.3% (Table 4).

Discussion

Isofetamid is the latest broad-spectrum member of the SDHIs registered for use on strawberry in Florida. Other SDHIs available for strawberry include boscalid, penthiopyrad, fluxapyroxad, and fluopyram. In this study, isofetamid showed high efficacy inhibiting growth of *B. cinerea* isolates resistant to the other SDHIs. However, a few isolates resistant to isofetamid that were also resistant to boscalid, penthiopyrad, and fluopyram appeared during the second season of monitoring. It has been shown in previous studies that resistance levels to penthiopyrad are similar to fluxapyroxad (Amiri et al. 2014). Thus, we chose to use only penthiopyrad in this study.

The initial step to monitor for the selection of fungicide resistance in pathogen populations is to determine the baseline sensitivity using isolates collected prior to widespread use of the fungicide. To our knowledge, this is the first study to determine the baseline sensitivity of *B. cinerea* isolates from strawberry to isofetamid, a thiophenecarboxamide fungicide (Xiong et al. 2015). Our results using isolates collected before isofetamid registration in 2015 showed a relatively wide range of EC₅₀ values. However, 83% of the strawberry isolates tested were at the lower end of the baseline (≤ 0.15 μ g/ml), similar to mycelium growth inhibition assays reported for isofetamid baseline in grapevine *B. cinerea* isolates (Piqueras et al. 2014).

Baselines with wide EC₅₀ ranges have also been reported for other SDHI fungicides such as penthiopyrad, fluopyram, and boscalid with *B. cinerea* isolates from table grape (Vitale et al. 2016) and

for other pathogens such *Alternaria alternata* (Avenot et al. 2014). The relatively wide EC₅₀ ranges can be related to genetic variability resulting in different responses among pathogen populations to the fungicide. The number of isolates can also be a factor for a wide sensitivity range, although Thomas et al. (2012) previously reported a baseline sensitivity in *Didymella bryoniae* using a similar number of isolates ($n = 71$) as used in our study but obtained narrow EC₅₀

Table 3. Identification of succinate dehydrogenase subunit B (SDH-B) mutations in *Botrytis cinerea* isolates

Isolate ^z	Phenotype ^x		Translated protein sequences ^y	
	Boscalid	Isofetamid	230	272
15-1000	HR	S	PSYWWNSEEYL	MSLYRCRTILNCS
17-02	HR	S	PSYWWNSEEYL	MSLYRCRTILNCS
17-51	HR	S	PSYWWNSEEYL	MSLYRCRTILNCS
17-63	HR	S	PSYWWNSEEYL	MSLYRCRTILNCS
17-103	HR	S	PSYWWNSEEYL	MSLYRCRTILNCS
17-109	HR	S	PSYWWNSEEYL	MSLYRCRTILNCS
17-241	HR	S	PSYWWNSEEYL	MSLYRCRTILNCS
15-1025	HR	S	PSYWWISEEYL	MSLYRCHTILNCS
15-1063	HR	S	PSYWWISEEYL	MSLYRCHTILNCS
16-32	HR	S	PSYWWISEEYL	MSLYRCHTILNCS
16-58	HR	S	PSYWWISEEYL	MSLYRCHTILNCS
16-88	HR	S	PSYWWISEEYL	MSLYRCHTILNCS
16-94	HR	S	PSYWWISEEYL	MSLYRCHTILNCS
17-10	HR	S	PSYWWISEEYL	MSLYRCHTILNCS
17-39	HR	S	PSYWWISEEYL	MSLYRCHTILNCS
17-94	HR	MR	PSYWWISEEYL	MSLYRCHTILNCS
17-99	HR	S	PSYWWISEEYL	MSLYRCHTILNCS
17-111	HR	MR	PSYWWISEEYL	MSLYRCHTILNCS
17-158	HR	S	PSYWWISEEYL	MSLYRCHTILNCS
17-211	HR	S	PSYWWISEEYL	MSLYRCHTILNCS
17-222	HR	MR	PSYWWISEEYL	MSLYRCHTILNCS
17-223	HR	S	PSYWWISEEYL	MSLYRCHTILNCS
17-234	HR	S	PSYWWISEEYL	MSLYRCHTILNCS

^x Resistance phenotypes obtained in this study for boscalid and isofetamid. S = sensitive, MR = moderately resistant, and HR = highly resistant.

^y Fragments of the translated protein sequences after alignment showing replacement of asparagine (N) by isoleucine (I) and histidine (H) by arginine (R) at codons 230 and 272, respectively.

^z *B. cinerea* isolates collected during the 2015–2016 and 2016–2017 strawberry seasons from different strawberry nurseries and Florida farms.

Fig. 3. Frequency of *Botrytis cinerea* isolates ($n = 565$) collected during two strawberry seasons from different nurseries and Florida fields, showing fungicide resistance (FR) to five succinate dehydrogenase inhibitor (SDHI) fungicides Boscalid, Penthiopyrad, Fluopyram, Benzovindiflupyr, and Isofetamid represented as Bos, Pent, Fluo, Benz, and Iso, respectively. S = sensitive, 1FR = resistance to one fungicide, 2FR = resistance to two fungicides, 3FR = resistance to three fungicides, 4FR = resistance to four fungicides, and 5FR = resistance to five fungicides.

Table 4. Efficacy of isofetamid on *Botrytis cinerea* isolates with different sensitivity levels

Isolate ^v	Genotype ^w	Phenotype ^x	Disease incidence (%) ^y	Conidial inhibition (%) ^z
05-26	WT	S	0.0 a	100.0 a
17-97		S	0.0 a	100.0 a
17-94	N230I	MR	55.6 b	89.9 b
17-222		MR	72.1 bc	91.3 bc
17-111		MR	77.0 c	70.7 cd

^v Isolates collected before and after isofetamid registration in 2015 in Florida. Isolates 17-94, 17-111, and 17-222 were found to be resistant in the fungicide sensitivity screening in this study.

^w Mutation N230I in the SDH-B subunit. WT = wild type.

^x Resistance phenotypes: S = sensitive and MR = moderately resistant.

^y Percentage of infected fruit showing Botrytis fruit rot symptoms relative to nontreated control. Data consisted of the mean of 48 values for each isolate from two experimental assays. Values followed by the same letter in the same column are not significantly different after analysis of variance and least significant difference test at $P \leq 0.05$.

^z Percentage of conidial inhibition by isofetamid relative to nontreated control. Data consisted of the mean 24 values for each isolate obtained from two experimental assays. Values followed by the same letter in the same column are not significantly different after analysis of variance and least significant difference tests at $P \geq 0.05$.

ranges for penthiopyrad and boscalid. Thus, the high genetic variability of *B. cinerea* (Williamson et al. 2007) might be the main contributing factor for the wide EC₅₀ range of our baseline isolates.

The first SDHI fungicides were released in 1966 (Leroux 2007), but a large spectrum of molecules considered highly effective against BFR were released after 2003. The rapid increase of SDHI use in combination with their mode of action targeting a single site makes them susceptible to selection for resistance. Different levels of fungicide resistance to the first broad-spectrum SDHI, boscalid, was previously reported to be conferred by the mutations H272R, H272Y, N230I, and P225F in *B. cinerea* isolates from Florida strawberry fields (Amiri et al. 2014). Esterio et al. (2015) and Yin et al. (2011) found the same mutations on *B. cinerea* isolates resistant to boscalid from table grape in Chile and apple in Washington, respectively. Boscalid has been used for the longest time among the SDHIs registered on strawberry, and resistance to this compound was observed soon after its registration (Amiri et al. 2014). For this reason, boscalid was used to compare SdhB mutations in this study.

Boscalid completely failed to inhibit mycelial growth of isolates harboring P225F mutation conferring high EC₅₀ values on in vitro assays (Amiri et al. 2014). Similar results were obtained for other SDHIs such as benodanil, biazafen, fenfuram, isopyrazam, fluxapyroxad, penthiopyrad, and fluopyram (Amiri et al. 2014; Veloukas et al. 2013). The P225F mutation also conferred high levels of resistance to isofetamid with EC₅₀ values over 500 µm/ml. Our results agree with previous findings indicating that this mutation is a great threat to the SDHI fungicides; however, this substitution has rarely been detected in field isolates (Veloukas et al. 2014). The mutations H272R and H272Y confer moderate to high levels of resistance to boscalid (Lalève et al. 2014; Veloukas et al. 2011) and are usually the most frequently found in *B. cinerea* strawberry isolates as reported by Fernández-Ortuño et al. (2012) and Leroux et al. (2010). The lack of fitness penalties especially for H272R represent a possible explanation for their persistence within populations (Veloukas et al. 2014), but these mutations failed to confer resistance to isofetamid in *B. cinerea* isolates in our study. Interestingly, N230I, a mutation of lower occurrence, showed moderate resistance in five isolates that carried the mutation. This contrasts with Veloukas et al. (2013), who reported in vitro growth inhibition of isolates with N230I mutation using fluopyram and other SDHIs, but isofetamid was not tested. Furthermore, Amiri et al. (2014) found that N230I conferred high to very high resistance to boscalid and fluxapyroxad, and different levels of resistance to penthiopyrad and fluopyram from moderate to very high resistance. These results support ours, considering that this mutation was found in resistant isolates to relatively new SDHI fungicides at the time, excluding boscalid.

In accordance with our SdhB phenotypic characterization, isolates with moderate resistance to isofetamid found in the 2016–2017 season were confirmed to have a substitution at codon 230 of asparagine by isoleucine (N230I). On average, these isolates showed a BFR incidence of 68.2% on strawberry sprayed with isofetamid and 84.0% inhibition of conidial production. Isolates with the same mutation caused 100% disease when sprayed with boscalid, fluopyram, and fluxapyroxad with the corresponding conidial inhibition percentages of 71.9, 57.4, and 72.2% (Amiri et al. 2014). Additional sequencing of the SdhB subunit for 20 randomly selected resistant isolates from the monitoring of resistance to SDHIs also identified the N230I mutation in 65% of the isolates, but they did not always confer resistance to isofetamid. Thus, based on these results, we hypothesize that the ability of *B. cinerea* to develop more than one mutation to an independently targeted site (Veloukas et al. 2014) might be a contributing factor for N230I mutation to confer resistance to isofetamid. Another interesting observation based on our results is that the resistant populations might be undergoing a genotypic predominance shift where H272R/Y is decreasing while N230I is increasing in Florida fields, which might explain the increasing resistance frequencies to fluopyram. However, future investigation is needed to better understand the effect of this mutation on fungicide sensitivity.

Monitoring of SDHI fungicide resistance frequencies was conducted for isolates collected from strawberry nurseries and Florida

farms for two consecutive seasons. Isofetamid, the newest SDHI fungicide, showed no resistance during 2015–2016 and 1.8% ($n = 3$) frequency of moderately resistant phenotype for the following season, but only on isolates collected from Florida fields. High frequency of sensitivity (100%) to this fungicide was also reported by Hu et al. (2018) against *B. cinerea* and *B. fragariae* isolates resistant to fungicides of one, two, three, four, or five chemical classes, likewise to other studies showing no resistance on *B. fragariae* (Dowling et al. 2018) and *B. mali* (Cosseboom et al. 2018). Furthermore, isofetamid has been reported to control *B. cinerea* effectively on table grape in Chile based on in vitro assays (Piqueras et al. 2014). The high in vitro effectiveness of isofetamid against *Botrytis* spp. according to our study confirms its high field efficacy compared with other SDHI fungicides (Cordova et al. 2015). Isofetamid is the second SDHI carrying a phenethylamide derivative (Jeanmart et al. 2016), which might explain the effective control observed in our and in other studies. However, an increase of resistance frequency was observed in our study to fluopyram, the first SDHI with the same characteristics as isofetamid. Therefore, the limited use of isofetamid is essential to preserve its efficacy.

In the second season (2016–2017), penthiopyrad and fluopyram resistance was observed at high frequencies in the two locations and only in Florida farms, respectively. Overall, a high frequency of *B. cinerea* resistance was observed to boscalid (93.9%) similar to that reported by Amiri et al. (2013, 2014) in Florida fields (85.4 and 100%) and by Oliveira et al. (2017) for nursery isolates (89.6%). Our results also agree with *B. cinerea* boscalid resistance on strawberry in the Carolinas (Fernández-Ortuño et al. 2012) and other hosts such as raspberry, grapevine, sweet cherry, and ornamental flowers in Germany (Rupp et al. 2017). However, Hu et al. (2016) found a much lower resistance frequency to boscalid in a study involving 12 states, not including Florida. The contradiction among studies could indicate the prevalence of boscalid resistance in Florida fields despite the fact that this fungicide is no longer used by commercial growers. On the other hand, this discrepancy could be explained by the different method used to conduct the experiments and the use of symptomatic strawberry fruit in our study versus asymptomatic flowers by Hu et al. (2016) for *B. cinerea* isolation. The same authors also reported very low resistance frequencies for penthiopyrad and fluopyram (<8% for each), similar to the results of Oliveira et al. (2017), which contradicts the corresponding frequencies obtained in this study, 59.1 and 30.6%, respectively. A previous report by Amiri et al. (2014) concluded that penthiopyrad rapidly selects for resistance in *B. cinerea*, which could explain our results in comparison with previous studies and the difference observed within the seasons evaluated.

Fluopyram resistance in our study was relatively high compared with that mentioned above, but its efficacy against BFR is higher compared with penthiopyrad (Zuniga et al. 2017). However, based on our results, resistance management of this fungicide needs improvement to preserve its efficacy. Benzovindiflupyr, an SDHI fungicide not yet registered for Botrytis fruit rot control on strawberry was also evaluated for resistance to *B. cinerea*. Overall frequency of resistance to this fungicide was low (25.7%) based on the conidial germination assay, indicating high activity against the pathogen. Our results agree with those of Hu et al. (2016) with the same effect on mycelial growth of strawberry isolates and to both conidial and mycelial inhibition of other pathogens such as *Venturia inaequalis* (Villani et al. 2016).

Fungicide-resistant (FR) phenotypes among the SDHI tested were also observed in this study. Resistant isolates to one fungicide were found at higher frequency (35%) compared with 2FR, 3FR, and 4FR, and 5FR isolates were observed at very low frequencies (0.5%). A previous study with *B. cinerea* isolates from nursery transplants reported 2FR occurred most commonly followed by 3FR (Oliveira et al. 2017). However, Amiri et al. (2013) found 2FR was the second highest after 3FR in *B. cinerea* isolates collected from fruiting fields. In agreement with these authors, *B. cinerea* resistance to more than one fungicide seems to occur at a higher rate. This might be due to the ability of the pathogen to develop more than one mutation related to a specific targeted site (Veloukas et al. 2014).

This study emphasizes the importance of resistance management of old and new SDHI fungicides, considering this group represents one of the most effective in managing BFR. Single-site fungicides should always be alternated or tank-mixed with multisite fungicide (Amiri et al. 2019) to minimize the selection of resistant populations. Reducing the use of fungicides with rapid selection for resistance such as penthiopyrad and limiting applications of other single-site fungicides such as fluopyram and isofetamid to only during bloom periods or following the Strawberry Advisory System (Cordova et al. 2017a; Pavan et al. 2017), and restricting applications in nurseries, can help preserve the efficacy of these fungicides. Finally, periodic evaluation of SDHI fungicides is advised to monitor resistance frequencies and predominant genotypes of SdhB mutations in *B. cinerea* from strawberry fields.

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