Resistance to the SDHI Fungicides Boscalid, Fluopyram, Fluxapyroxad, and Penthiopyrad in *Botrytis cinerea* from Commercial Strawberry Fields in Spain

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Abstract

Gray mold, caused by the necrotrophic fungus *Botrytis cinerea*, is one of the most economically important diseases of strawberry. Gray mold control involves the application of fungicides throughout the strawberry growing season; however, *B. cinerea* isolates resistant to multiple classes of site-specific fungicides have been recently reported in the Spanish gray mold population. Succinate dehydrogenase inhibitors (SDHI) constitute a relatively novel class of fungicides registered for gray mold control representing new alternatives for strawberry growers. In the present study, 37 *B. cinerea* isolates previously characterized for their sensitivity to boscalid and amino acid changes in the SdhB protein were used to determine the effective concentration that reduces mycelial growth by 50% (EC₅₀) to fluopyram, fluxapyroxad, and penthiopyrad. The present study was also conducted to obtain discriminatory doses to monitor SDHI fungicide resistance in 580 *B. cinerea* isolates collected from 27 commercial fields in Spain during 2014, 2015, and 2016. The EC₅₀ values ranged from 0.01 to >100 µg/ml for fluopyram, <0.01 to 4.19 µg/ml for fluxapyroxad, and, finally, <0.01 to 59.65 µg/ml for penthiopyrad. Based on these results, as well as findings from a previous publication, the discriminatory doses chosen to examine

sensitivities to boscalid, fluopyram, fluxapyroxad, and penthiopyrad were 100, 15, 1, and 6 µg/ml, respectively. Over the course of the 3-year monitoring period, the overall frequencies of resistance to the four SDHI were 56.9, 6.9, 12.9, and 24.6%, respectively. The frequency of boscalid-resistant isolates decreased from 73 to 41% over the years; however, the fluopyram-resistant isolates increased from 5 to 10% after 1 year of registration. Four SDHI resistance patterns were observed in our population, which included patterns I (30%; resistance to boscalid), II (13.8%; resistance to boscalid and penthiopyrad), III (5.7%; boscalid, fluxapyroxad, and penthiopyrad), and IV (7.9%; resistance to boscalid, fluopyram, fluxapyroxad, and penthiopyrad). Patterns I and II were associated with the amino acid substitutions H272R and H272Y; pattern III was associated only with the H272Y mutation; and, finally, pattern IV was associated with the N230I allele in the SdhB subunit. For gray mold management, it is suggested that the simultaneous use of boscalid and penthiopyrad should be limited to one application per season; however, fluxapyroxad and, especially, fluopyram could be used as valid SDHI alternatives for gray mold control, although they should be applied with caution.

Gray mold, caused by *Botrytis cinerea* Pers., is one of the most devastating diseases for more than 230 plant species worldwide, including ornamental plants, vegetables, and fruit such as strawberry (Jarvis 1977). Due to the absence of commercially available gray-mold-resistant cultivars of strawberry, control must be achieved through the intensive application of fungicides throughout the strawberry growing season. However, according to the Fungicide Resistance Action Committee (FRAC; www.frac.info), *B. cinerea* has been categorized as a high-risk pathogen due to the development of fungicide resistance. Consequently, the effective chemical management of gray mold has been hindered by the development of resistant isolates. Resistance to multiple site-specific fungicides in *B. cinerea* has been documented in many countries worldwide (Amiri et al. 2013; Bardas et al. 2010; Fernández-Ortuño et al. 2014; Leroux et al. 2013; Weber and Hahn 2011).

Sterol biosynthesis and quinone-oxidase inhibitors (QoI) are two of the most relevant classes of fungicides for disease control in commercial agriculture; however, in terms of new compounds produced and registered on the market, the succinate dehydrogenase inhibitors (SDHI) is the class with the fastest growth (Sierotzki and Scalliet 2013). Due to the limited disease and application spectrum of the “first-generation” SDHI (known as carboxamide fungicides) to date, 18 SDHI active ingredients belonging to eight chemical classes with

a broader spectrum of fungal activity are available for control of fungal plant pathogens (FRAC). SDHI have a single-site mode of action. They inhibit cell energy production by binding to the ubiquinone-binding pocket of succinate dehydrogenase (complex II), which is part of the tri-carboxylic cycle and is linked to mitochondrial electron transfer in the fungal respiration pathway (Ruprecht et al. 2009). The target enzyme consists of four subunits: a hydrophilic flavoprotein (SdhA) and an iron-sulfur protein (SdhB) that form the membrane-peripheral domain and the two subunits (SdhC and SdhD), membrane-hydrophobic proteins that anchor the enzyme complex to the inner mitochondrial membrane. The ubiquinone-binding site comprises amino acids from subunits B, C, and D (Hägerhäll 1997).

Like other fungicides with a single-site mode of action and widespread use, SDHI are classified as medium to high risk for resistance development (FRAC). Resistance to the “new generation” of SDHI (mainly boscalid) has been documented shortly after their registration in field isolates of several plant pathogens and hosts (Amiri et al. 2014; Avenot and Michailides 2009; Avenot et al. 2008, 2012; Ishii et al. 2011; Stammler et al. 2014; Villani et al. 2016; Vitale et al. 2016; Yang et al. 2015; Yin et al. 2011). Different target site mutations in the subunits B, C, or D have been linked to confer reduced sensitivity to SDHI in several fungal plant pathogen species (FRAC). With regard to *B. cinerea*, different mutations of the *sdhB* gene result in the amino acid substitution of proline by phenylalanine, histidine, leucine, or threonine (P225F, -H, -L, or -T, respectively) at codon 225 (Amiri et al. 2014); substitution of a conserved asparagine residue by isoleucine (N230I) at codon 230 (Amiri et al. 2014; Hu et al. 2016); and, finally, the substitution of histidine by either tyrosine, leucine, or arginine (H272Y, -L, or -R, respectively) at codon 272 (Fernández-Ortuño et al. 2012; Leroux et al. 2010; Veloukas et al. 2011; Yin et al. 2011). These changes have been associated with the loss of SDHI efficacy.

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Only one mutation has been reported in the *sdhD* subunit, leading to a substitution of histidine by arginine at codon 132 (allele H132R) (Leroux et al. 2010).

In Spain, only two SDHI fungicides, boscalid and fluopyram, are marketed for strawberry gray mold control in combination with the QoI fungicides pyraclostrobin (Signum; BASF Española S.A.) and trifloxystrobin (Luna Sensation; Bayer CropScience S.L.), respectively. Penthioapyrad and fluxapyroxad are additional highly efficacious SDHI fungicides that are expected to soon be registered for gray mold control of strawberry in Spain. A recent study carried out in Spain has documented resistant isolates of *B. cinerea* to the six classes of site-specific fungicides registered for strawberry gray mold control (Fernández-Ortuño et al. 2016). This study showed evidence of high levels of resistance and a decline in efficacy of fungicides such as pyraclostrobin, boscalid, cyprodinil, and fenhexamid (Fernández-Ortuño et al. 2016), emphasizing the need for new, effective fungicides for strawberry growers. The objectives of the present study were to (i) establish sensitivity to fluopyram, fluxapyroxad, and penthiopyrad; (ii) determine the frequency of resistance and cross-resistance patterns to boscalid,

fluopyram, fluxapyroxad, and penthiopyrad in *B. cinerea* isolates sampled from strawberry fields over three seasons; and (iii) investigate the point mutations in the *sdhB* gene associated with resistance.

Materials and Methods

Fungal isolates. In total, 37 single-spore isolates of *B. cinerea* were examined to determine discriminatory doses for fluopyram, fluxapyroxad, and penthiopyrad (Table 1). Of these, 33 isolates were collected in Spain and characterized for their sensitivity to boscalid using a spore germination assay and amino acid changes in SdhB in a previous study (Fernández-Ortuño et al. 2016). The four remaining isolates (JEY2013.2, 82-10, 112-9, and BALL1) originated from the United States and were kindly provided by Dr. Guido Schnabel and used as controls. The American isolates were also characterized for their sensitivity and mutations in the *sdhB* gene to boscalid, fluopyram, fluxapyroxad, and penthiopyrad in recent studies (Table 1) (Fernández-Ortuño et al. 2015; Hu et al. 2016).

For SDHI monitoring studies, 580 *B. cinerea* single-spore isolates were collected between 2014 and 2016 from 27 commercial strawberry

Table 1. Isolate name, origin, year of isolation, absence or presence of point mutations in the succinate dehydrogenase iron-sulfur protein (*SdhB*) gene, and sensitivity phenotype of *Botrytis cinerea* isolates^a

Name	Origin	Year	Allele	Phenotype in two assays			
				Spore germination	Mycelial growth (EC ₅₀ µg/ml) ^c		
				Boscalid ^b	Fluopyram	Fluxapyroxad	Penthiopyrad
Spain							
12	Moguer	2013	H272	S	S (1.96)	SS (0.01)	S (0.63)
29	Moguer	2013	H272	S	S (1.34)	SS (<0.01)	S (0.25)
62	Lucena del Puerto	2013	H272	S	S (2.13)	S (0.11)	S (1.02)
87	Almonte	2013	H272	S	S (1.16)	S (0.14)	S (0.70)
100	Almonte	2013	H272	S	S (0.95)	SS (<0.01)	S (0.44)
115	Lepe	2013	H272	S	S (0.77)	SS (<0.01)	S (0.11)
149	Almonte	2013	H272	SS	S (1.15)	SS (0.04)	S (0.26)
175	La Redondela	2013	H272	S	S (1.14)	SS (<0.01)	SS (0.07)
193	P. de la Frontera	2013	H272	SS	S (1.60)	SS (0.03)	SS (0.02)
196	P. de la Frontera	2013	H272	SS	S (1.01)	SS (<0.01)	SS (0.01)
241	Isla Cristina	2013	H272	SS	S (1.20)	SS (<0.01)	SS (0.03)
244	Almonte	2014	H272	S	S (1.79)	S (0.55)	S (0.46)
290	Moguer	2014	H272	S	S (0.32)	SS (<0.01)	SS (<0.01)
312	Cartaya	2014	H272	S	S (0.41)	SS (0.03)	S (0.18)
337	Lepe	2014	H272	S	S (0.96)	S (0.35)	S (0.26)
3	Moguer	2013	H272R	R	S (2.05)	S (0.24)	R (3.55)
75	Lucena del Puerto	2013	H272R	R	S (0.36)	S (0.51)	S (0.24)
80	Lucena del Puerto	2014	H272R	R	S (1.67)	SS (0.02)	R (3.51)
84	Almonte	2014	H272R	R	S (2.10)	S (0.14)	R (5.52)
90	Almonte	2014	H272R	R	S (3.69)	SS (0.03)	R (3.46)
93	Almonte	2013	H272R	R	S (2.29)	S (0.32)	R (6.28)
190	P. de la Frontera	2014	H272R	R	S (1.12)	SS (0.04)	R (3.24)
223	Isla Cristina	2014	H272R	R	S (2.08)	SS (<0.01)	R (10.95)
229	Isla Cristina	2014	H272R	R	S (2.08)	SS (<0.01)	R (5.08)
41	Moguer	2014	H272Y	R	S (3.09)	SS (0.03)	R (20.01)
45	Moguer	2013	H272Y	R	S (0.92)	S (0.25)	R (3.45)
51	Moguer	2014	H272Y	R	S (3.30)	SS (0.06)	R (22.08)
117	Lepe	2013	H272Y	R	SS (0.01)	R (1.92)	R (8.69)
133	Almonte	2014	H272Y	R	S (3.36)	SS (0.06)	R (39.80)
146	Almonte	2014	H272Y	R	S (5.62)	S (0.37)	R (59.65)
155	Almonte	2014	H272Y	R	S (4.84)	S (0.30)	R (38.31)
237	Isla Cristina	2014	H272Y	R	S (3.09)	S (0.13)	R (14.78)
313	Cartaya	2014	H272Y	R	S (0.24)	R (2.06)	R (25.93)
United States							
JEY2013.2	South Carolina	2013	H272Y	R	S (0.68)	R (1.81)	R (10.47)
82-10	n/a ^d	2015	P225F	R	HR (>100)	R (4.19)	R (41.24)
112-9	n/a	2015	N230I	R	R (31.00)	R (2.04)	R (32.60)
BALL1	South Carolina	2014	N230I	R	R (22.25)	R (1.78)	R (29.05)

^a SS = highly sensitive, S = sensitive, R = resistant, and HR = highly resistant.

^b Boscalid was tested at 1 and 50 µg/ml, as previously described Weber and Hahn (2011).

^c EC₅₀ = effective concentration that reduces mycelial growth by 50%.

^d Not available.

fields in southern Spain. Among the fields, 25 were obtained from different locations in the province of Huelva, including Almonte, Cartaya, Isla Cristina, Moguer, La Redondela, Lepe, Lucena del Puerto, Palos de la Frontera, and San Bartolome de la Torre. The two remaining commercial fields were located in the province of Malaga in Algarrobo-Costa. Details related to the number of fields, isolates, cultivars, and fungicide applications are given in Table 2. For all isolates, the conidia were collected from symptomatic fruit using a sterile cotton swab and single-spore isolates were obtained as previously described (Fernández-Ortuño et al. 2012).

Sensitivity to fluopyram, fluxapyroxad, and penthiopyrad and determination of discriminatory doses. A commercial formulation of fluopyram (Luna Privilege; Bayer CropScience S.L.) was used in this study. Commercial formulations of fluxapyroxad and penthiopyrad were kindly provided by the manufacturers BASF SE and DuPont, respectively. Fungicide stock solutions of 10,000 µg/ml were prepared by dissolving the fungicides in sterilized distilled water. The solutions were stored at 4°C until use. Potato dextrose agar (PDA), yeast bacto acetate agar (YBA), and yeast extract agar (YEA) were the media used for SDHI sensitivity tests. For sensitivity testing, fungicide stock solutions were diluted in sterile distilled water and then added to cooling (approximately 50°C) autoclaved PDA, YBA, and YEA to obtain the following active ingredient concentrations: 0.01, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, and 100 µg/ml. The control medium was not supplemented with fungicides. Three replicates were used per concentration.

To determine the effective concentration that reduces mycelial growth by 50% (EC₅₀) in comparison with the control, 90-mm-diameter Petri dishes containing fungicide-amended or unamended media were inoculated with a nonsporulating mycelium plug (10 mm in diameter) from a 3-day-old culture of each isolate grown on PDA medium. Each colony diameter (minus the diameter of the inoculation plug) was measured in two perpendicular directions after 3 days

of incubation at 22°C in the dark. The EC₅₀ values were graphically determined from the log-transformation of percentages of inhibition and regression against the natural logarithm of fungicide concentrations. The experiment was repeated twice.

Based on the EC₅₀ values obtained for all representative isolates listed in Table 1, a single discriminatory concentration that could be applied in mycelial growth assays to distinguish sensitive from resistant isolates for each fungicide was determined for field monitoring studies. Tests were conducted in wells (15 mm in diameter) of 24-well plates (6 by 4 wells, 12.5 by 8.5 by 2 cm; Sarstedt). Conidia from sporulating PDA plates were transferred directly to the center of each well using a sterile wooden toothpick. Inoculated plates were incubated at 22°C and diametric colony growth was visually assessed in each well.

Determination of mutations in the *sdhB* gene. The identification of *sdhB* mutations was carried out on isolates that were resistant to any SDHI. To extract DNA, 50 *B. cinerea* isolates were grown on PDA plates for 4 days at 22°C. Genomic DNA was extracted from 10 to 20 mg of mycelium, as previously described by Chi et al. (2009). The primer pair IpBcBeg and IpBcEnd2 was used to amplify a fragment of the *sdhB* gene of approximately 950 bp in length (Leroux et al. 2010) containing the codons 225, 230, and 272. Polymerase chain reaction (PCR) was performed in a final volume of 25 µl containing 50 ng of fungal genomic DNA, 0.5 µl of 10 mM dNTP, 0.5 µl of 10 µM each primer, 2.5 µl of 10× Thermo Pol Reaction Buffer, and 0.125 µl of Taq DNA Polymerase (New England Biolabs). PCR amplifications were achieved in a MyCycler thermal cycler (Bio-Rad Laboratories) following an initial preheating at 95°C for 3 min; followed by 35 cycles consisting of 30 s denaturation at 95°C, 30 s of annealing at 60°C, and 1 min of extension at 72°C; and a final extension stage of 4 min at 72°C.

Aliquots of 10 µl of the PCR products were separated on 1% agarose gels stained with RedSafe Nucleic Acid (Intron Biotechnology),

Table 2. Origin, field code, year of isolation, number of single-spore isolates (*N*), strawberry cultivar, and fungicide applications in the strawberry fields where the *Botrytis cinerea* isolates were collected

Location	Field code	Year	<i>N</i>	Strawberry cultivar ^a	Fungicide applications ^b
Huelva					
Almonte	Fresmol	2014	27	Fortuna	AP
	Guaral	2014	24	Fortuna	n/a
	Bora	2015	24	n/a	none
	Doana	2015	12	n/a	n/a
Cartaya	Arfa	2015	9	n/a	n/a
	Cart1	2016	16	n/a	DMI; H(2A)P
	Cart2	2016	15	n/a	n/a
Isla Cristina	Alseca	2014	25	Benicia; Fortuna; Sabrina	KRI
Moguer	Celar	2014–15	41	n/a	n/a
	Ocesas	2014–15	36	n/a	n/a
	Mogu1	2016	12	n/a	DMI; DPC; H(2A)P; sulfur
	Mogu2	2016	19	n/a	n/a
	Mogu3	2016	15	n/a	DMI
	Mogu4	2016	17	n/a	DMI; DPC; H(2A)P; sulfur
	Mogu5	2016	19	n/a	n/a
	Mogu6	2016	17	n/a	AN; DMI; QoI; sulfur
	Mogu7	2016	13	n/a	AN; DMI; QoI; sulfur
	Mogu8	2016	20	n/a	DMI
La Redondela	Mareno	2014–15	34	Candonga	<i>B. subtilis</i> ; KRI
Lepe	Adva	2014–15	48	n/a	n/a
Lucena del Puerto	Alares	2014	29	Benicia; San Andreas	n/a
	Luerto	2016	18	n/a	n/a
Palos de la Frontera	Cuvas	2014	18	Fortuna	n/a
	Fresquito	2014	23	Fortuna	n/a
San Bartolome de la Torre	Fresor	2015	11	n/a	n/a
Malaga					
Algarrobo-Costa	Perasca	2016	15	Camarosa	n/a
	Aulia	2016	23	Camarosa	None

^a Abbreviation: n/a = not available.

^b AN = aza-naphthalenes, AP = anilinopyrimidines, DPC = dinitrophenyl crotonates, DMI = demethylation inhibitors, H(2A)P = hydroxy-(2-amino-)pyrimidines, KRI = keto-reductase inhibitors, and QoI = quinone outside inhibitors.

run in 1× Tris-Borate-EDTA buffer, and exposed to UV light to visualize DNA fragments. PCR products were purified using the ExoSAP-IT PCR purification kit (USB Corporation) according to the manufacturer's instructions and subsequently sequenced in both directions at Macrogen Europe. Sequences were analyzed using the computer sequence analysis software DNASTAR (DNASTAR, Inc.).

Statistical analysis. To test whether there were differences in resistance to the four tested fungicides, we implemented a generalized linear mixed model (GLMM) in which sensitivity to fungicide was set as a binomial response variable (with values equal to either 0 for sensitive isolates or 1 for resistant isolates) and fungicide treatment was set as a fixed predictor factor with four levels (i.e., each of the fungicides). Because isolates were collected in different locations and years, both variables were included in the model as random factors. Similarly, for those sites where we had collected isolates for three consecutive years, we compared them through a GLMM in terms of the frequency of resistance among the sampled years. In this case, we compared the same binomial response variable (with values equal to either 0 for sensitive isolates or 1 for resistant isolates) with treatment (four levels, i.e., each fungicide), year, and the interaction term year–treatment as fixed factors. To detect possible covariation in the occurrence of resistance between the used fungicides, we performed a Pearson correlation analysis. All statistical analyses were conducted using SAS (version 9.3; SAS Institute Inc.).

Results

Sensitivity and determination of discriminatory doses to fluopyram, fluxapyroxad, and penthiopyrad. In this study, a mycelial growth inhibition assay was used to determine the sensitivities of 33 *B. cinerea* isolates collected from different locations in southern Spain to the SDHI fungicides fluopyram, fluxapyroxad, and penthiopyrad. After 3 days of incubation at 22°C, very little to no growth was observed on YBA and YEA media at the fungicide concentrations tested (data not shown). Similar results were observed after 5 and 7 days of incubation (data not shown). Based on these observations, PDA was the medium used for SDHI sensitivity tests, as described previously (Amiri et al. 2014; Kim and Xiao 2010; Song et al. 2016; Zhang et al. 2007).

For fluopyram, all the isolates were sensitive. Among them, 1 isolate was highly sensitive, with an EC₅₀ value of 0.01 µg/ml, and 32 isolates had EC₅₀ values of 0.24 to 5.62 µg/ml (mean = 1.81 µg/ml; Tables 1 and 3). For fluxapyroxad, 19 isolates were highly sensitive, with EC₅₀ values of <0.01 to 0.06 µg/ml (mean = 0.02 µg/ml); 12 isolates were sensitive, with EC₅₀ values between 0.11 and 0.55 µg/ml (mean = 0.28 µg/ml); and, finally, 2 isolates were fluxapyroxad resistant, with EC₅₀ values of 1.92 and 2.06 µg/ml (mean = 1.99 µg/ml; Tables 1 and 3). For penthiopyrad, the 33 Spanish isolates studied were classified as highly sensitive (5 isolates), sensitive (11 isolates), or resistant (17 isolates). The EC₅₀ values for the different groups ranged from <0.01 to 0.07 µg/ml (mean = 0.02 µg/ml), 0.18 to 1.02 µg/ml (mean = 0.41 µg/ml), and 3.24 to 59.65 µg/ml (mean = 16.13 µg/ml), respectively (Tables 1 and 3).

As expected, the EC₅₀ values obtained in this study for fluopyram, fluxapyroxad, and penthiopyrad for the four American isolates were in agreement with their fungicide sensitivity phenotypes characterized in previous studies (Fernández-Ortuño et al. 2015; Hu et al. 2016) (Table 1).

Based on the EC₅₀ results obtained for the 37 *B. cinerea* isolates (33 Spanish and 4 American), the discriminatory concentrations used to distinguish sensitivity from resistant isolates during the 3-year monitoring studies were 15, 1, and 6 µg/ml for fluopyram, fluxapyroxad and penthiopyrad, respectively. For boscalid (Cantus; BASF), the discriminatory concentration chosen for sensitivity was 100 µg/ml, as previously described (Amiri et al. 2014).

Three-year monitoring of SDHI resistance in *B. cinerea* on strawberry. After 3 days of incubation of the inoculated plates testing the discriminatory doses described previously, isolates resistant to the four SDHI were distinguished from sensitive isolates because they showed a 100% diametrical growth with a very dense mycelium, similar to the growth on the unamended control wells

(Fig. 1A and B). The majority of the sensitive isolates presented absence of growth (Fig. 1C) for the four SDHI. Few isolates presented a thin diametrical growth for boscalid, fluopyram, and penthiopyrad (Fig. 1D and E). These isolates were considered sensitive in our assay because they were sensitive for SDHI when they were tested in other culture media at different discriminatory doses (Hu et al. 2016; Weber and Hahn, 2011) and analyzed for the absence of mutations in *sdhB* (data not shown).

The GLMM clearly showed significant differences in the resistance rates to the four compared fungicides. The overall frequencies of resistance for the 580 *B. cinerea* isolates to boscalid, fluopyram, fluxapyroxad, and penthiopyrad were 56.9, 6.9, 12.9, and 24.6%, respectively (Fig. 2A). The comparison of resistance among the localities sampled in three consecutive years revealed a consistent pattern in the observed fungicide resistance. For the 3 years, the highest resistance was observed for boscalid and penthiopyrad; however, for fluopyram and fluxapyroxad, resistance was consistently low (Fig. 2B). In 2014, 73.4, 5.0, 14.3, and 31.6% of the 239 isolates analyzed were resistant to boscalid, fluopyram, fluxapyroxad, and penthiopyrad, respectively. In 2015, the frequencies of resistance were lower for boscalid (56.2%) and penthiopyrad (20.5%) and were similar for fluopyram (5.3%) and fluxapyroxad (13.1%) compared with the previous year. Finally, during the 2016 strawberry growing season, the frequencies of resistance were even lower for boscalid (41.2%) and fluxapyroxad (11.2%), similar for penthiopyrad (21.8%), and higher for fluopyram (10.4%) (Fig. 2B).

Resistance to boscalid was widespread and found in all locations (Fig. 3). Resistance to fluopyram was found all the locations except for Alares, Aulia, Bora, Doana, Fresor, Fresquito, Mogu6, and Ocesas (Fig. 3); and resistance to fluxapyroxad was found in all locations except for Alares, Aulia, Doana, Fresquito, and Mogu6 (Fig. 3). Resistance to penthiopyrad was also presented in the majority of the fields analyzed but was not found in Aulia, Doana, and Mogu6 (Fig. 3).

Cross-resistance among SDHI fungicides. The Pearson correlation test carried out after analyzing the SDHI phenotypes by screening 580 *B. cinerea* isolates (Table 4) revealed a weak positive correlation between boscalid and penthiopyrad ($r = 0.52$, $P < 0.0001$), between fluxapyroxad and penthiopyrad ($r = 0.65$, $P < 0.0001$) and, finally, between fluxapyroxad and fluopyram ($r = 0.73$, $P < 0.0001$).

SDHI resistance patterns in *Botrytis cinerea* isolates collected over 3 years. The sensitivity of each SDHI tested at one discriminatory dose divided the 580 isolates into four different fungicide resistance patterns. Pattern I represented isolates resistant only to boscalid. Pattern II represented isolates resistant to the SDHI boscalid and penthiopyrad. Pattern III represented isolates resistant to the three SDHI boscalid, fluxapyroxad, and penthiopyrad. Finally, pattern IV represented isolates resistant to all SDHI tested in this study (boscalid, fluopyram, fluxapyroxad, and penthiopyrad). In total, 174 (30.0%), 80 (13.8%), 33 (5.7%), and 46 (7.9%) isolates represented patterns I, II, III, and IV, respectively (Table 5). With only two exceptions, pattern I was found in all locations studied; pattern II was found in 18 of 27 fields; pattern III was found in 11 fields, including Adva, Alseca, Arfa, Bora, Cuvas, Fresmol, Fresor, Guara, Mareno, Ocesas,

Table 3. Effective concentration that reduces mycelial growth by 50% (EC₅₀) values for Spanish *Botrytis cinerea* isolates highly sensitive (SS), sensitive (S), and resistant (R) to fluopyram, fluxapyroxad, and penthiopyrad

Fungicide	Phenotype	EC ₅₀ (µg/ml)	
		Range	Mean
Fluopyram	SS	0.01	0.01
	S	0.24–5.62	1.81
Fluxapyroxad	SS	<0.01–0.06	0.02
	S	0.11–0.55	0.28
	R	1.92–2.06	1.99
Penthiopyrad	SS	<0.01–0.07	0.02
	S	0.18–1.02	0.41
	R	3.24–59.65	16.13

Fig. 1. Mycelium growth of *Botrytis cinerea* isolates on **A**, unamended control and potato dextrose agar amended at one discriminatory dose to the four succinate dehydrogenase inhibitors. **B**, Resistant isolates were distinguished from **C**, **D**, and **E**, sensitive isolates in their ability to fill the entire well with a very dense mycelium.

Fig. 2. Frequency of *Botrytis cinerea* isolates resistant to the four succinate dehydrogenase inhibitors **(A)** collected from 27 strawberry fields during the 3-year monitoring studies **(B)**.

and Perasca (Table 5); and pattern IV (resistance to four SDHI) was found in 19 fields (Table 5).

Detection of *sdhB* mutations in *B. cinerea* isolates. Some isolates representing the resistance patterns I, II, III, and IV were selected randomly for *sdhB* sequencing (Table 6). Sequencing analysis reported nucleotide alterations in the 50 isolates that had demonstrated resistance to at least one SDHI fungicide. The amino acid substitution H272R was found in 14 isolates resistant to boscalid (pattern I) and 5 isolates resistant to boscalid and penthiopyrad (pattern II); however, the amino acid change H272Y was also detected in 2 and 9 isolates belonging to patterns I and II, respectively. The 10 isolates analyzed with resistance to boscalid, fluxapyroxad, and penthiopyrad (pattern III) only carried the substitution from histidine to tyrosine (H272Y). Finally, the 10 isolates resistant to the four SDHI showed the amino acid substitution of N230I (Table 6).

Discussion

In the present study, the SDHI fungicide resistance profiles of the gray mold population of strawberry in Spain were investigated. Boscalid was the first member of the new-generation SDHI registered in 2007 in Spain against gray mold on strawberry, and resistance to this active ingredient was recently recorded (Fernández-Ortuño et al. 2016); however, this study is the first report of resistance to fluopyram, fluxapyroxad, and penthiopyrad in Spain. Reports of boscalid resistance in *B. cinerea* isolates from several hosts were documented soon after its worldwide commercial use in apple (Kim and Xiao 2010), grape (Leroch et al. 2011; Leroux et al. 2010), kiwifruit (Bardas et al. 2010), strawberry (Fernández-Ortuño et al. 2012; Stammler et al. 2008), and vegetable greenhouses (Zhang et al. 2007). In our study, after analyzing the evolution of boscalid-resistant isolates during the 3-year monitoring studies, it was observed that the frequency of resistant isolates decreased from 73 to 41%. According to the majority of the Spanish strawberry producers, this result could be due to the reduction of the number of Signum applications in their fields because other active ingredients such as fenhexamid (Teldor; Bayer) and the prepacked mixture of cyprodinil and fludioxonil (Switch; Syngenta España S.A.) have become the most frequently used fungicides during the last few years (Anonymous 2008, 2015). In addition, the recent introduction of fluopyram + trifloxystrobin could also have been contributing to the reduction of the frequency of isolates resistant to boscalid. This fact is in agreement with previous findings observed after the discontinuous use of boscalid + pyraclostrobin in the control of gray mold on apple, strawberry, and grape (Fernández-Ortuño et al. 2014; Kim and Xiao 2011; Walker et al. 2013).

Low frequencies of resistance were observed for fluopyram, the recently registered SDHI in Spain during the summer of 2015; however, after 1 year of use, there was a slight increase in resistant isolates from 5 to 10%. The low levels of resistance found in this study, which are in agreement with previous publications (Amiri et al. 2014; Hu et al. 2016; Veloukas and Karaoglanidis 2012; Vitale et al. 2016), could be explained by the fact that the molecule fluopyram has an active residue (benzamide moiety) different from boscalid, fluxapyroxad, and penthiopyrad that binds residues from two subunits (SdhB and SdhC) in mitochondrial complex II. This binding interaction suggests that amino acid alterations in both subunits may be necessary to affect the efficacy of this fungicide (Fraaije et al. 2012). In *Mycosphaerella graminicola*, fluopyram selected for mutants carrying residue changes in the SdhC subunit more frequently than in the subunit SdhB (Fraaije et al. 2012) which, at the moment, are the most frequently described in *B. cinerea*. In *Alternaria alternata*, it has recently been published that the mutation H134R in SdhC provided high levels of resistance to fluxapyroxad but was also associated with the highest resistance levels to fluopyram (Yang et al. 2015). Thus, the low levels of fluopyram-resistant isolates in Spain may be due to the type of *sdhB* mutations and their frequencies that, at the moment, are dominated by the H272R and H272Y alleles (Fernández-Ortuño et al. 2016) not associated with fluopyram resistance (Amiri et al. 2014; Hu et al. 2016).

Although fluxapyroxad and penthiopyrad have not yet been registered for gray mold control of strawberry in our country, the sensitivity of these fungicides was established because both are in the process of registration for commercial use to control several plant diseases in different crops, because it has already been used in other countries worldwide (Anonymous 2016). The fields tested in this study had never been exposed to any treatment with these SDHI; however,

Table 4. Pearson correlation coefficients (*r*) and *P* values (<0.0001) obtained after analyzing the frequencies of resistance to boscalid, fluopyram, fluxapyroxad, and penthiopyrad in 580 isolates of *Botrytis cinerea*

Fungicide	Fungicide			
	Boscalid	Fluopyram	Fluxapyroxad	Penthiopyrad
Boscalid	1.00	...	...	...
Fluopyram	0.25	1.00	...	...
Fluxapyroxad	0.34	0.73	1.00	...
Penthiopyrad	0.52	0.47	0.65	1.00

Fig. 3. Frequency of *Botrytis cinerea* isolates resistant to boscalid, fluopyram, fluxapyroxad, and penthiopyrad collected from 27 locations in southern Spain.

resistance to fluxapyroxad and penthiopyrad was presented in the gray mold population and always in combination with resistance to boscalid. The overall frequencies of resistance to fluxapyroxad (12.9%) and penthiopyrad (24.6%) in our study were higher compared with Hu et al. (2016), who reported resistance frequencies of 5.5 and 7.4% to both fungicides during the 2014–15 strawberry growing seasons in the United States. However, our results were in agreement with Amiri et al. (2014), who documented similar frequencies of resistance, including data from strawberry fields never sprayed with penthiopyrad, which was the case in the present study. Cross-resistance between boscalid and penthiopyrad was previously described in strawberry isolates of *B. cinerea* (Amiri et al. 2014; Hu et al. 2016) and other fungal species such as *A. alternata* (Avenot and Michailides 2009; Yang et al. 2015), *A. solani* (Mallik et al. 2014), *M. graminicola* (Fraaije et al. 2012), *Corynespora cassiicola*, and *Podosphaera fusca* (Ishii et al. 2011). *B. cinerea* isolates resistant to boscalid, fluxapyroxad, and penthiopyrad have been previously described in strawberry fields in the United States with similar

Table 5. Number and frequency (%) of *Botrytis cinerea* isolates sensitive to four succinate dehydrogenase inhibitors and belonging to four different resistance patterns

Fields	N ^a	Sensitive	Number per resistance pattern (%) ^b			
			I	II	III	IV
Adva	48	20 (41.7)	10 (20.8)	6 (12.5)	7 (14.6)	5 (10.4)
Alares	29	10 (34.5)	12 (41.4)	7 (24.1)	0 (0.0)	0 (0.0)
Alseca	25	1 (4.0)	17 (68.0)	4 (16.0)	1 (4.0)	2 (8.0)
Arfa	9	4 (44.5)	1 (11.1)	0 (0.0)	2 (22.2)	2 (22.2)
Aulia	23	16 (69.6)	7 (30.4)	0 (0.0)	0 (0.0)	0 (0.0)
Bora	24	10 (41.7)	5 (20.8)	8 (33.3)	1 (4.2)	0 (0.0)
Cart1	16	7 (43.8)	4 (25.0)	0 (0.0)	0 (0.0)	5 (31.2)
Cart2	15	8 (53.4)	2 (13.3)	0 (0.0)	0 (0.0)	5 (33.3)
Celar	41	21 (51.3)	11 (26.8)	6 (14.6)	0 (0.0)	3 (7.3)
Cuvas	18	3 (16.8)	7 (38.9)	6 (33.3)	1 (5.5)	1 (5.5)
Doana	12	10 (83.3)	2 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
Fresmol	27	12 (44.5)	4 (14.8)	4 (14.8)	4 (14.8)	3 (11.1)
Fresor	11	3 (27.3)	6 (54.5)	1 (9.1)	1 (9.1)	0 (0.0)
Fresquito	23	7 (30.4)	12 (52.2)	4 (17.4)	0 (0.0)	0 (0.0)
Guaral	24	3 (12.6)	14 (58.3)	0 (0.0)	5 (20.8)	2 (8.3)
Luerto	18	13 (72.3)	3 (16.7)	1 (5.5)	0 (0.0)	1 (5.5)
Mareno	34	13 (38.2)	13 (38.2)	5 (14.7)	2 (5.9)	1 (3.0)
Mogu1	12	7 (58.4)	1 (8.3)	0 (0.0)	0 (0.0)	4 (33.3)
Mogu2	19	5 (26.3)	3 (15.8)	9 (47.4)	0 (0.0)	2 (10.5)
Mogu3	15	12 (80.0)	0 (0.0)	2 (13.3)	0 (0.0)	1 (6.7)
Mogu4	17	9 (52.9)	2 (11.8)	5 (29.4)	0 (0.0)	1 (5.9)
Mogu5	19	9 (47.4)	7 (36.8)	0 (0.0)	0 (0.0)	3 (15.8)
Mogu6	17	15 (88.2)	2 (11.8)	0 (0.0)	0 (0.0)	0 (0.0)
Mogu7	13	11 (84.6)	0 (0.0)	1 (7.7)	0 (0.0)	1 (7.7)
Mogu8	20	9 (45.0)	5 (25.0)	5 (25.0)	0 (0.0)	1 (5.0)
Ocesas	36	3 (8.4)	21 (58.3)	5 (13.9)	7 (19.4)	0 (0.0)
Perasca	15	6 (40.0)	3 (20.0)	1 (6.7)	2 (13.3)	3 (20.0)
Total	580	247 (42.6)	174 (30.0)	80 (13.8)	33 (5.7)	46 (7.9)

^a Number of isolates.

^b Pattern I = resistant to boscalid; II = resistant to boscalid and penthiopyrad; III = resistant to boscalid, fluxapyroxad, and penthiopyrad; IV = resistant to boscalid, fluopyram, fluxapyroxad, and penthiopyrad.

Table 6. Amino acid substitutions in the SdhB protein of *Botrytis cinerea* isolates representing four succinate dehydrogenase inhibitor resistance patterns

Pattern	N ^a	Genotype		
		H272R	H272Y	N230I
I Boscalid	16	14	2	0
II Boscalid and penthiopyrad	14	5	9	0
III Boscalid, fluxapyroxad, and penthiopyrad	10	0	10	0
IV Boscalid, fluopyram, fluxapyroxad, and penthiopyrad	10	0	0	10

^a Number of isolates studied.

frequency (3.2%) (Hu et al. 2016) to the 5.7% frequency reported in the present study.

The four resistance patterns detected in this study were correlated with different point mutations in the *sdhB* gene. The amino acid substitutions H272R and H272Y were associated with resistance to boscalid (pattern I) and resistance to boscalid and penthiopyrad (pattern II). Resistance to boscalid, fluxapyroxad, and penthiopyrad (pattern III) was associated exclusively with the amino acid change H272Y, and resistance to boscalid, fluopyram, fluxapyroxad, and penthiopyrad (pattern IV) was associated with the N230I allele. These results are in agreement with previous studies where different levels of resistance (low to strong), represented with a range of EC₅₀ values, were associated with different point mutations in the *sdhB* subunit. In the present study, only one discriminatory dose was tested for each SDHI and, for this reason, a specific mutation was able to confer more than one resistance pattern. In future studies, it could be interesting to test at least two discriminatory doses in order to distinguish in more detail the frequencies of the alleles that confer resistance to SDHI. For boscalid, Yin et al. (2011) observed that the four boscalid-resistant phenotypes found in the gray mold population from apple were not associated with a particular type of point mutation (H272R versus H272Y) because both genotypes were present in isolates that represented the four phenotypes. Veloukas et al. (2011) and Leroux et al. (2010) reported that the mutations H272R and H272Y were associated with moderate levels of resistance to boscalid. For boscalid and penthiopyrad, Hu et al. (2016) described that the majority of the strawberry *B. cinerea* isolates resistant to boscalid and penthiopyrad carried the H272Y allele; however, one isolate contained the H272R allele in the SdhB subunit. In other fungal species, the arginine (R) codon was also found to confer resistance to boscalid and penthiopyrad but at a lower level than the tyrosine (Y) codon in the equivalent residue of the SdhB homologous protein (Avenot and Michailides 2010; Ishii et al. 2011; Van den Biggelaar 2010). According to other authors, resistance to boscalid, fluxapyroxad, and penthiopyrad in *B. cinerea* and other fungal species, including *A. alternata*, *Didymella bryoniae*, *C. cassiicola*, and *P. xanthii*, was associated exclusively with the amino acid substitution H272Y (Amiri et al. 2014; Avenot et al. 2012; Hu et al. 2016; Ishii et al. 2011; Leroux et al. 2010). Finally, the substitution N230I was only correlated with resistance to the four SDHI in our *B. cinerea* population, and this result is in agreement with previous studies in *B. cinerea* (Amiri et al. 2014; Hu et al. 2016).

In view of the high frequencies of boscalid-resistant isolates in Spain, new SDHI alternatives are necessary for disease management. Based on our results, penthiopyrad application in crops where resistance to boscalid is widespread will lead to an increase in the selection pressure and frequencies of the resistant population. For this reason, the simultaneous use of boscalid and penthiopyrad in the strawberry spray programs should be avoided, limiting their applications to one per season, as previously suggested (Hu et al. 2016). Interestingly, in contrast to penthiopyrad, fluxapyroxad and especially fluopyram should play a key role in gray mold management; however, these fungicides should be used with caution in spray programs because they could favor the selection of novel mutations or help to increase the frequency of mutations that, at the moment, occur at low frequencies. For example, the H272L and P225F and -L mutations were documented to confer cross-resistance between boscalid and fluopyram (Veloukas et al. 2013; Walker et al. 2013) in *B. cinerea* and, actually, they were not observed in our population. Another point to consider is the presence of other mechanism of resistance not based on point mutations but, instead, on multidrug resistance. Therefore, the sensitivity of the *B. cinerea* population to SDHI fungicides should be frequently monitored in order to report shifts in sensitivities. In addition, rotation and mixture with other site-specific or multisite fungicides such as thiram and captan should be included to improve disease management in the gray mold population, as shown in a recent study (Menzel et al. 2016).

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