Fungicide Resistance Profiles of *Botrytis cinerea* Isolates From Michigan Vineyards and Development of a TaqMan Assay for Detection of Fenhexamid Resistance

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Abstract

Botrytis cinerea on grapes causes bunch rot at both pre- and postharvest stages, in which losses can reach up to 100%. Chemical control primarily relies on the prophylactic use of site-specific fungicides. Repeated applications of these products raise the risk of fungicide resistance development in *B. cinerea* populations, which can result in disease control failures. To determine the extent of resistance, *B. cinerea* isolates were collected from grape clusters in the northwest and southwest grape growing regions of Michigan in 2014 and 2018 ($n = 115$ and 125 , respectively). These isolates were phenotyped using discriminatory doses of eight fungicides to determine the levels of resistance. Fungicide resistance increased from 2014 to 2018, mostly affecting the active ingredients fenhexamid, fluopyram, and iprodione. *B. cinerea* isolates resistant to multiple fungicides were detected in 2014 and 2018, with a higher frequency of

resistance in 2018. TaqMan real-time PCR has been developed to detect *B. cinerea* fungicide resistance to fenhexamid and to differentiate the *erg27* F412S/I/V alleles. The TaqMan assay was tested for sensitivity, specificity, and reproducibility on purified DNA and infected grape tissue samples. Our data provide essential information to growers about the efficacy for *B. cinerea* control using the available botryticides. Furthermore, the developed fenhexamid markers will be transferred to diagnostic clinics to assist growers in the management of bunch rot before resistance-related control failures occur.

Keywords: bunch rot, disease management, fruit, fungi, multiple drug resistance, pathogen detection, site-specific fungicides, small fruits, techniques, *Vitis* spp.

Botrytis bunch rot caused by *Botrytis cinerea* Pers. is a major fungal disease of grapevines in temperate climates and is responsible for significant economic losses worldwide (Ficke et al. 2002; Hartman and Kaiser 2008). The pathogen starts to infect flowers at bloom and remains quiescent, most likely at the receptacles of the fruit, until berries begin to ripen (Gindro et al. 2005; Holz et al. 2003; McClellan and Hewitt 1973; Viret et al. 2004). During fruit ripening, the pathogen infects berries through injured or cracked tissue, aggressively rots the fruits, and produces gray masses of conidia that can spread throughout the field by wind, causing successive infections (Hartman and Kaiser 2008; Holz et al. 2007; McClellan and Hewitt 1973; Puhl and Treutter 2008; Weber and Hahn 2011). Although various cultural practices, such as leaf removal to increase canopy airflow, are available in vineyards and play important roles in disease management programs, control of bunch rot significantly depends on the application of fungicides.

Several site-specific synthetic fungicides with different modes of action are used to control *B. cinerea* on grapes (Leroux et al.

2002b; Saito et al. 2019) and on other economically important crops such as strawberry (Rupp et al. 2017), blueberry (Saito et al. 2016), hops (Pfeiffer 2016), and vegetables (Myresiotis et al. 2007). These fungicides include quinone outside inhibitors (QoIs; Fungicide Resistance Action Committee [FRAC] code 11), benzimidazoles (FRAC 1), phenylpyrroles (FRAC 12), succinate dehydrogenase inhibitors (SDHIs; FRAC 7), anilinothiazoles (FRAC 9), dicarboximides (FRAC 2), and hydroxylanilides (FRAC 17). However, repeated application of fungicides with the same mode of action has led to the development of resistance to one or more fungicides and consequently makes the control of gray mold challenging (Amiri et al. 2014; Fernández-Ortuño et al. 2015; Leroux et al. 2002b; Rupp et al. 2017; Saito et al. 2019; Veloukas et al. 2014). Fungicide resistance of *B. cinerea* isolates has been reported worldwide in various fruits and vegetables (De Miccolis Angelini et al. 2014; Esterio et al. 2011; Leroux et al. 2011; Panebianco et al. 2015; Rupp et al. 2017; Veloukas et al. 2014; Yin et al. 2012). Understanding fungicide resistance of *B. cinerea* populations and their prevalence could help develop effective control programs. However, there is no information about fungicide resistance in *B. cinerea* populations from Michigan vineyards.

Fenhexamid, a hydroxylanilide derivative, was registered in the United States under the trade name Elevate in 1999 for use in grapes, strawberries, ornamentals, and other crops. Fenhexamid inhibits 3-ketoreductase involved in the C-4 demethylation complex during ergosterol biosynthesis (Debieu et al. 2001). Growers have favored the application of fenhexamid in gray mold management in the United States because of its unique mode of action and good efficacy. However, resistance to fenhexamid in *B. cinerea* has been reported in several crops worldwide, including grapes (Fillinger et al. 2008; Grabke et al. 2013; Grez et al. 2017; Moorman et al. 2012; Myresiotis et al. 2007; Pokorny et al. 2016; Rupp et al. 2017; Saito et al. 2019; Yin et al. 2016). In *B. cinerea*, resistance to fenhexamid is associated with point mutations within the *erg27* gene that encodes the 3-ketoreductase (Albertini and Leroux 2004; Esterio et al. 2011; Fillinger et al. 2008; Grabke et al. 2013; Leroux et al. 2002a). Various amino acid substitutions in the target protein have been reported to confer resistance to

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fenhexamid in *B. cinerea*. For instance, amino acid substitution at codons 63 (T63I), 412 (F412S/I/V/C), and 496 (T496R) was detected in Hydr3⁺ isolates that show a high to moderate level of resistance to fenhexamid (Albertini and Leroux 2004; Fillinger et al. 2008; Grabke et al. 2013). In Hydr3⁻ isolates with weak to moderate resistance to fenhexamid, numerous amino acid substitutions, including F26S, L195F, V309M, A314V, S336C, N369D, L400F, L400S, P238S, I199L, and Y408S, and a deletion of P298 were found (Esterio et al. 2011; Fillinger et al. 2008).

The objectives of this study were to (i) investigate fungicide resistance phenotyping of *B. cinerea* populations collected from different vineyards and years across Michigan, (ii) determine genotyping of fenhexamid-resistant isolates of *B. cinerea*, and (iii) develop a rapid molecular-based assay to detect fenhexamid resistance in *B. cinerea* populations.

Materials and Methods

***B. cinerea* isolate collection.** In total, 240 *B. cinerea* isolates were collected from Michigan vineyards in 2014 and 2018 ($n = 115$ and 125 isolates, respectively). Vineyards were located in Southwest or Northwest Michigan. Eight and 10 grape clusters with bunch rot disease symptoms were collected from each vineyard in 2014 and 2018, respectively. Grape clusters were sampled from grape cultivars commonly grown in Michigan such as Chardonnay, Pinot Noir, Riesling (*Vitis vinifera*), Niagara (*V. labrusca*), and Aurore, Chancellor, and Vignoles (*Vitis* interspecific hybrid).

Conidia of *B. cinerea* from infected grape clusters were spread on water agar media (15 g of agar in 500 ml of distilled water), and the plates were then incubated at room temperature for approximately 16 h. A single conidium of *B. cinerea* was isolated and transferred onto another plate containing 20% clarified V8 agar media (100 ml of V8 juice, 1 g of CaCO₃, 10 g of agar, and 400 ml of distilled water). To store isolates of *B. cinerea*, the glass fiber method was used with slight modifications (Peever and Milgroom 1992). Briefly, isolates were subcultured on water agar (7.5 g of agar in 500 ml of distilled water) with approximately 1-cm² sterile glass fiber pieces. Seven to 10 days later when the fungal mycelia covered the glass fiber pieces, they were collected in sterile coin envelopes. The envelopes were then placed in magenta boxes without lids, and the boxes were kept in a biosafety hood to air dry for up to 2 days. After 2 days, a drying agent (Drierite) was added to the bottom of the boxes, and the boxes were then sealed and stored at -20°C.

Fungicide preparation. A total of eight fungicides of seven different classes were used in this study. Five technical-grade fungicides were obtained from Sigma-Aldrich: boscalid (PESTANAL, analytical standard, 99.5%), pyraclostrobin (PESTANAL analytical standard, 99.5%), fluopyram (PESTANAL, analytical standard, 100.0%), fludioxonil (PESTANAL, analytical standard, 99.5%), and thiabendazole (PESTANAL, analytical standard, 98.6%). All technical-grade active ingredients (a.i.) were dissolved in 100% acetone to prepare a stock solution of 1,000 ppm except fluopyram, for which the stock solution was adjusted to 500 ppm. All stock solutions were stored in the dark at 4°C. A working solution with a concentration of 200 ppm was prepared for each of the five fungicides. The other three fungicides in the study were commercial products: Vanguard (75% cyprodinil a.i.; Syngenta, Basel, Switzerland), Rovral (41.6% iprodione a.i.; Bayer Crop Science, Monheim am Rhein, Germany), and Elevate 50WDG (50% fenhexamid a.i.; UPL Limited, Mumbai, India). All commercial fungicides were dissolved in sterile distilled water to prepare the 1,000-ppm stock solution. All stock solutions from commercial products were stored in the dark at 4°C.

***B. cinerea* fungicide resistance phenotyping.** The 240 *B. cinerea* isolates were screened for sensitivity to the eight above-mentioned fungicides (fludioxonil, thiabendazole, boscalid, pyraclostrobin, fenhexamid, iprodione, fluopyram, and cyprodinil) following the protocol originally developed by Weber and Hahn (2011) and modified by Saito et al. (2016) with slight modification in this study. Fludioxonil, fenhexamid, thiabendazole, and iprodione were tested on 1% malt extract agar (MEA) media. Pyraclostrobin was tested on 1% MEA with salicylhydroxamic acid, an alternative oxidase inhibitor, added

to the media at a final concentration of 100 µg/ml (Mondal et al. 2005; Wise et al. 2008). Cyprodinil was tested on 0.5% sucrose media to avoid the interference of amino acids from the assay (Hou et al. 2018; Myresiotis et al. 2007). To avoid the interference of sugars (Leroux 2007) with fungicidal activity, boscalid and fluopyram were tested on 0.5% yeast extract agar. After autoclaving, all media were placed in a water bath at 50°C. Once the media temperature reached 50°C, the fungicide was added to its corresponding type of media. Two milliliters of each medium with eight different fungicides was poured into a well of a 12-well cell plate. Two milliliters of each medium without fungicide was used as the control. The 12-well cell plates were placed in zip-top plastic bags (Ziploc) and stored at 4°C. All plates were used within 2 weeks of storage. Control isolates were used to ensure fungicide inhibitory activity during the experiment.

A conidial suspension was prepared from 7- to 14-day-old cultures grown on 20% clarified V8 agar media. Conidia were harvested from the culture plates by scraping the surface of the plate with a sterile toothpick, then suspended in approximately 1 ml of sterile 0.01% Tween 20 solution. The conidial suspension was filtered through sterile cheesecloth. The concentration of conidia in the suspension was quantified under a microscope using a hemocytometer and adjusted to 1.0×10^5 spores/ml. Spore suspensions were placed on ice until use. The 12-well cell plates were air-dried in a biosafety cabinet before use; 20 µl of conidial suspension of each isolate was added to each well of the plate, then plates were left for 10 min in the biosafety cabinet without a lid to air dry. The plates were incubated for 14 to 16 h in the dark at 20°C. After incubation, at least 20 conidia were examined for germination and germ tube elongation under a compound microscope using $\times 10$ magnification. *B. cinerea* isolates with known fungicide sensitivities to the eight fungicides were used in this study as control isolates (Saito and Xiao 2018; Saito et al. 2016, 2019). All experiments were performed twice.

A single discriminatory dose of each fungicide was used as described previously (Saito and Xiao 2018; Saito et al. 2016, 2019; Weber and Hahn 2011), in which fludioxonil and iprodione were tested at 0.1 and 5 ppm, respectively, while cyprodinil, fluopyram, boscalid, fenhexamid, and thiabendazole were tested at 1 ppm. Fungicide-resistant and fungicide-sensitive phenotypes were determined using the following criteria: an isolate was classified as resistant (R) if the germ tube length was >50% relative to the control growth, while an isolate was classified as sensitive (S) if the germ tube length was <50% relative to the control growth. Statistical *t* test analyses were conducted using R software (R Core Team 2017) to detect statistical differences in fungicide sensitivity between the two study years.

Fitness cost of *B. cinerea* resistance to fenhexamid. To investigate the fitness cost of *B. cinerea* isolates resistant to fenhexamid that carry different mutations, mycelial growth rates of 49 *B. cinerea* isolates resistant to fenhexamid were compared with those of 18 isolates sensitive to fenhexamid. Fenhexamid-resistant isolates that harbor different mutations of *erg27* include F412S ($n = 29$), F412I ($n = 12$), F412V ($n = 2$), T63I ($n = 1$), and N369S+P238S ($n = 5$). Of the 18 selected fenhexamid-sensitive isolates tested, two isolates had the mutation N369D+P238S. Mycelial growth rates were compared with sensitive isolates at three different temperatures: 10, 20, and 28°C. Agar plugs (5 mm) containing actively growing mycelium were excised with a sterile cork borer from the edge of 3-day-old potato dextrose agar (PDA) cultures maintained at 20°C, and the plug was placed upside down in the center of the PDA plate. Plates were then incubated in the dark at 10, 20, and 28°C. Three replicate plates were used for each isolate and each temperature. Colony diameter was measured in two perpendicular directions after 72 h of incubation. Pairwise comparisons were achieved using Tukey's honest significant difference test ($P = 0.05$) to determine significance between rates of growth among isolates. Statistical analyses were conducted using R statistical software (R Core Team 2017). In the statistical test, isolates with the same genotype were treated as replicates.

For more investigation of the fitness cost of isolates with different mutations and to clarify the relationship between the presence of

mutations N369D and P238S in resistant and sensitive isolates, a conidial germination and germ tube elongation assay was conducted for isolates having either N369D, P238S, or both mutations, in addition to two isolates with the F412V mutation, three isolates from each genotype of F412S and F412I, and four sensitive isolates with no mutations at different fenhexamid concentrations (0.1, 0.5, 1, 5, 10, 25, and 50 µg/ml). The conidial germination and germ tube elongation assay was conducted following the same procedure described above.

DNA extraction and *erg27* gene sequencing. Thirty-one and 49 *B. cinerea* isolates identified as sensitive and resistant to fenhexamid, respectively, based on the in vitro assay described above were used in this study to determine the molecular characteristics of *erg27*. Mycelia were collected from 1- to 2-week-old cultures that were grown on 20% clarified V8 agar media. Mycelia were lyophilized, then approximately 5 mg of tissue was ground using a TissueLyser (Qiagen, Valencia, CA). Automated DNA extraction was performed on the ground mycelia using the MagMax plant DNA isolation kit (Thermo Fisher Scientific, Waltham, MA) and processed on the KingFisher Flex purification system (Thermo Fisher Scientific). DNA samples were quantified using two methods to obtain accurate results. In the first method, DNA samples were processed with the Qubit 1X dsDNA HS assay kit (Thermo Fisher Scientific), and concentrations were then quantified by the Qubit 4 fluorometer (Thermo Fisher Scientific). In the second method, DNA samples were processed using PicoGreen Quant-iT DNA reagent and kits (Thermo Fisher Scientific), then the DNA concentration was determined using the Synergy HTX multimode reader (BioTek, Winooski, VT). DNA concentration was quantified using PicoGreen Quant-iT DNA reagent and kits (Thermo Fisher Scientific), then the DNA concentration was determined using the Synergy HTX multimode reader (BioTek). PCR was performed to amplify *erg27* in *B. cinerea* isolates using the oligonucleotide pair *erg27*Beg (forward) and *erg27*End (reverse) (Fillinger et al. 2008). Reaction was performed in a final volume of 50 µl containing 10× ThermoPol buffer, 1.25 units of Taq DNA polymerase (New England BioLabs, Ipswich, MA), 50 ng of DNA, 10 mM of dNTPs, and 10 µM of each primer. Thermocycling conditions included an initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 61°C for 30 s, and 68°C for 2 min, and a final extension at 68°C for 10 min. Reaction was performed on a C1000 Touch thermal cycler (Bio-Rad Laboratories, Hercules, CA). PCR product size was examined on 1% agarose gel with gelRed nucleic acid stain (Bio-Rad Laboratories), and appropriately sized PCR products were purified using the QIAquick PCR purification kit (Qiagen). Purified products were sequenced with five primers, *erg27*Beg, *erg27*End, *erg2000*up, *erg1800*down (Fillinger et al. 2008), and F412 forward (Grabke et al. 2013), using the ABI Prism BigDye terminator version 3.1 cycle sequencing kit and performed on the ABI 3730xl platform at the Research Technology Support Facility at Michigan State University.

***Erg27* gene sequence alignment and TaqMan probe design.** All sample consensus sequences were aligned using multiple alignment to *erg27* reference gene KP027845 (Yin et al. 2016) downloaded from GenBank. Sequence analyses were conducted using Geneious software 10.2.6 (Biomatters Ltd., Auckland, New Zealand). Generally, sequence alignments were screened for two single nucleotide polymorphisms (SNPs) known for association with high resistance to fenhexamid: T63I and F412I/V/S/C.

Specific primer pairs were designed to link with four dual-labeled probes to amplify a 306-bp amplicon. Probes were designed to specifically bind to the *erg27* wild allele (F) and three mutant alleles (S, I, V) at amino acid position 412. To multiplex the detection reaction, probes were labeled at the 5' end with either FAM, HEX, CAL Fluor Red 610, or Quasar 670. Probes and primers were ordered from LGC Biosearch Technologies (Novato, CA). The in silico probe sequence for allele F412C and primer sets and probe for the T63I mutation were designed in this study (Table 1). Sequences of amplification primers and probes developed in this study are shown in Table 1.

During the optimization of multiple allele detection and preservation of TaqMan assay specificity, the probe Bot_Fen-S labeled with HEX occasionally cross-reacted in the negative control. Therefore, an additional locked nucleic acid (LNA) Bot_Fen_LNA-S probe labeled with HEX was designed to be examined for no interference with reaction specificity in the multiplex detection assay. Six LNAs were added to the Bot_Fen-S probe sequence around and between the two SNPs (Table 1). Bot_Fen_LNA-S was ordered from Integrated DNA Technologies (Coralville, IA).

Optimization of the TaqMan assay for detection of fenhexamid resistance alleles. A TaqMan assay was performed to differentiate the four alleles at amino acid position 412 in *erg27* associated with high resistance to fenhexamid. Reaction was performed using Perfecta qPCR ToughMix (Quanta Biosciences, Gaithersburg, MD) and 10 ng of DNA (Supplementary Table S1). To determine the best reaction conditions, four primer concentrations (50, 300, 500, and 900 nM) in combination with different gradient annealing temperatures (Tms) ranging from 60 to 68°C were tested. Three replicates were tested for each combination. As a negative control, water was used instead of DNA template. A primer concentration of 300 nM and Tm at 66.7°C provided results with the highest specificity but not high sensitivity. The initial probe concentration used in the assay was 125 nM; however, assay sensitivity was improved by increasing the probe concentration to 250 nM. Since the probe Bot_Fen-S HEX was occasionally amplified in the negative control, 0.4× dimethyl sulfoxide (DMSO) was added to the reaction mixture to aim for a more specific and sensitive reaction. Using this reaction mixture, the TaqMan assay was conducted again with gradient Tm ranging from 62 to 66°C in the presence of 0.4× DMSO. Specific detection and no cross-reaction from the negative control was achieved using a Tm of 65°C. Our final optimized conditions for the TaqMan

Table 1. Amplification primers and quantitative PCR probes specific for detection of *Botrytis cinerea* fenhexamid resistance-associated mutations at amino acid residue 412 using the TaqMan assay

Name ^a	Sequence 5' to 3'	Length (bp)	Tm (°C) ^b	5' label	3' quencher	GC (%)
Amplification primer						
Bc_Fen_erg27F412F	CCTCTTCATGGTTCGATTGT	20	55.5			45.0
Bc_Fen_erg27F412R	CGTCCATGTTATCTAGAACATCG	23	56.9			43.5
Bc_erg27_T63I-F*	ATGAGCTCCAAGATCCCCGAA	21	61.0			52.4
Bc_erg27_T63I-R*	ATGAACCCGAGCCACCGTAT	20	61.3			55.0
TaqMan probe						
Bot_Fen-F	TCTACAGATTGATCTTCTACCTTGTAAGATG	31	60.6	FAM	BHQ-1	35.5
Bot_Fen-S	TCTACAGATTGATCTCTACCTTGTAAGATG	31	61.9	HEX	BHQ-1	38.7
Bot_Fen-I	TCTACAGATTGATCATCTACCTTGTAAGATG	31	60.7	CAL Fluor Red 610	BHQ-2	35.5
Bot_Fen-V	TCTACAGATTGATCGTCTACCTTGTAAGATG	31	62.3	Quasar 670	BHQ-2	38.7
Bot_Fen_LNA-S	ACAGATT+GA+TC+T+CC+TA+CCTTGTAAG	19	65.0	HEX	Iowa Black FQ	40.0
Bot_Fen-C*	TCTACAGATTGATCTGCTACCTTGTAAGATG	31	62.5	Quasar 705	BHQ-3	38.7
Bc_Fen-T63I*	AAGACACGATTCATAATCTCCCGCCT	26	64.0	CAL Fluor Red 610	BHQ-2	46.2

^a Asterisks denote in silico designed primers and probes for mutations reported in other studies of *Botrytis cinerea* fenhexamid resistance.

^b Tm = annealing temperature.

assay reaction mixture were performed in 20 µl that contained 300 nM of each primer pair, 250 nM of each probe, 10× Perfecta qPCR ToughMix, 0.4× DMSO, and 10 ng of DNA (Supplementary Table S1). The amplification quantitative PCR (qPCR) reaction was performed using the following thermal cycling conditions: initial denaturation at 95°C for 2 min, followed by 44 cycles of 95°C for 10 s and 65°C for 45 s; fluorometric data were collected at the end of each cycle (Supplementary Table S1). The assay was conducted using the CFX96 Touch Real-Time System (Bio-Rad Laboratories). All assay reactions were performed in triplicate.

A standard curve was made on a serial dilution of DNA of *B. cinerea* isolates with the four different genotypes. DNA concentrations were 5, 1, 0.5, 0.1, 0.05, 0.001, and 0.0001 ng. Reactions were performed in triplicate and water was used as the negative control. A

standard curve plot was created from these serial dilutions for each genotype. Slope and efficiency of the standard curve plot were automatically calculated in qPCR software (CFX96 Touch Real-Time System; Bio-Rad Laboratories).

Validation of the TaqMan assay. TaqMan assays were conducted under the conditions described above to detect mutant alleles of *erg27* at amino acid position 412 in 49 fenhexamid-resistant isolates. Thirty-one *B. cinerea* isolates with known sensitivity to fenhexamid were included in the test. A DNA sample of the closely related fungal pathogen, *Sclerotinia sclerotiorum*, was also tested in the TaqMan assay to determine assay specificity to *B. cinerea*.

In 2019, three grape clusters exhibiting bunch rot sporulation were collected from each of five different vineyards across Northwest and Southwest Michigan (two vineyards were sampled previously in 2014 and 2018, and three new vineyards were sampled in 2019). At one vineyard located in the southwest of the state, three clusters from three different grape cultivars were collected. Berries were collected from the clusters of every vineyard to include five berries with *Botrytis* sporulation, five berries with symptoms of bunch rot but not sporulation (symptomatic nonsporulated), and five berries that were healthy. Peels of these berries were collected by squeezing the fruit, then peels were stored in Falcon tubes at −20°C. The process of berry collection from the grape clusters was replicated three times for each berry tissue type (sporulated, symptomatic nonsporulated, and healthy). Examples of symptomatic nonsporulated berries were cracked berry, berry with a change in color, or berry with bird peck marks. Additionally, 10 samples with a mixture of leaves and vines with bunch rot symptoms were collected from each of three vineyards in California. All leaf and vine samples were collected in 1.5-ml tubes and stored at −20°C. Frozen samples of grape berry peels and grape leaves and vines that were collected from Michigan vineyards and California vineyards, respectively, were lyophilized and then 5 mg of tissue was used for DNA extraction. DNA was extracted from three replicates per sample following the procedure described above. The TaqMan assay was performed in triplicate on 25 extracted unquantified DNA samples under the same reaction conditions and thermal cycling parameters described. The 25 tested samples included grape leaves collected from four California vineyards ($n = 4$) and grape clusters collected from five Michigan locations ($n = 21$). Twenty-one samples were examined from Michigan grapes, which included three different berry tissues (sporulating, symptomatic nonsporulating, and healthy) for each of the four locations and the location with three cultivars. The negative control (no template) and two positive controls of samples with F and S alleles were included in the test.

Results

Frequency of resistance to site-specific fungicides in *B. cinerea* isolates in Michigan vineyards. Fungicide sensitivity was observed in *B. cinerea* isolates collected in 2014 and 2018 from vineyards in Northwest and Southwest Michigan. All *B. cinerea* isolates collected in 2014 and 2018 were sensitive to fludioxonil except one isolate that was collected in 2018 from a vineyard in Northwest Michigan. In 2014, 73.9, 91.3, 93, 3.4, 26.9, 3.47, and 62.6% of the isolates were resistant to thiabendazole, boscalid, pyraclostrobin, fenhexamid, iprodione, fluopyram, and cyprodinil, respectively; in 2018, 84.8, 95.2, 99.2, 38.4, 54.4, 30.4, and 69.6% of the isolates were resistant to these seven fungicides, respectively (Fig. 1). Statistical analysis results showed significant differences in fenhexamid, iprodione, and fluopyram resistance between 2014 and 2018, with P values of 0.01, 0.04, and 0.01, respectively. The rest of the fungicides were not significantly different between the two study years.

We compared the profile of fungicide sensitivity of *B. cinerea* isolates collected in Southwest and Northwest Michigan vineyards between the two study years. Results indicate that in 2014, the isolates collected from Southwest Michigan vineyards had lower frequencies of resistance to all fungicides compared with those from Northwest Michigan vineyards, except fenhexamid and cyprodinil (Fig. 2A). In 2018, isolates from Southwest Michigan generally had lower resistance frequencies to all fungicides, while resistance

Fig. 1. Comparison of fungicide resistance frequency in *Botrytis cinerea* between two sampling years 2014 and 2018, where isolates were collected from infected grape clusters in Northwest and Southwest Michigan vineyards. The number of isolates was 115 and 125 in 2014 and 2018, respectively. Fd = fludioxonil, Th = thiabendazole, Bo = boscalid, Py = pyraclostrobin, Fn = fenhexamid, Ip = iprodione, Fp = fluopyram, and Cy = cyprodinil.

Fig. 2. Comparison of fungicide resistance frequency in *Botrytis cinerea* between Northwest and Southwest Michigan vineyards. **A**, 2014 ($n = 115$). **B**, 2018 ($n = 125$). Fd = fludioxonil, Th = thiabendazole, Bo = boscalid, Py = pyraclostrobin, Fn = fenhexamid, Ip = iprodione, Fp = fluopyram, and Cy = cyprodinil.

to fludioxonil and fenhexamid was detected only in Northwest Michigan vineyards (Fig. 2B).

We also compared the profile of *B. cinerea* isolate fungicide sensitivity in four locations (three in Southwest Michigan and one in Northwest Michigan), where we sampled from those same fields in both years. In 2018, resistance to fluopyram, but not to iprodione, was detected in Southwest Michigan location 1, and resistance frequency to boscalid and thiabendazole declined (Fig. 3A). In Southwest Michigan location 2, resistance to boscalid and cyprodinil decreased over both years, while resistance to iprodione increased and resistance to fenhexamid and fluopyram disappeared (Fig. 3B). In Southwest Michigan location 3, resistance to boscalid, fluopyram and pyraclostrobin increased, while resistance to thiabendazole, iprodione, and cyprodinil decreased in 2018 (Fig. 3C). In Northwest Michigan location 4, resistance to fenhexamid and fluopyram developed in 2018 (Fig. 3D), while resistance to thiabendazole and iprodione increased. No resistance to fludioxonil was observed in any of the four locations in both years (Fig. 3).

Multiple fungicide resistance (MFR) was detected in *B. cinerea* populations from Michigan vineyards. The *B. cinerea* populations

tended to shift to phenotypes simultaneously resistant to more fungicides with different modes of action (Supplementary Fig. S1). MFR frequency increased in 2018 to five different classes of fungicides and resistance to six and seven different fungicides developed in the 2018 population. Resistance to all eight tested fungicides was not detected in the populations in 2014 and 2018. Twenty-three fungicide-resistant phenotypes were detected in the *B. cinerea* population collected in 2018, while 13 fungicide-resistant phenotypes were detected in the 2014 population (Fig. 4). Twelve phenotypes were commonly observed in *B. cinerea* populations of both years (Fig. 4). Simultaneous resistance to pyraclostrobin, boscalid, thiabendazole, cyprodinil, iprodione, and fenhexamid was the most predominant MFR phenotype, with 20% of the 2018 population; in 2014, the most predominant phenotype was resistance to a combination of fungicides comprising pyraclostrobin, boscalid, thiabendazole, and cyprodinil, with 35% of the population (Fig. 4). We observed that population sensitivity to all eight tested fungicides decreased from 6.1 in 2014 to 0.8 in 2018 (Fig. 4).

Genotypes of fenhexamid resistance in *B. cinerea* populations. We amplified and sequenced *erg27* in 49 fenhexamid-resistant

Fig. 3. Fungicide resistance frequency of *Botrytis cinerea* isolates collected from the same locations in 2014 and 2018. **A, B, and C,** Vineyards in Southwest Michigan. **D,** Vineyard in Northwest Michigan. The number of isolates was as follows: A, 2014 ($n = 10$) and 2018 ($n = 10$); B, 2014 ($n = 9$) and 2018 ($n = 10$); C, 2014 ($n = 15$) and 2018 ($n = 10$); and D, 2014 ($n = 8$) and 2018 ($n = 10$). Fd = fludioxonil, Th = thiabendazole, Bo = boscalid, Py = pyraclostrobin, Fn = fenhexamid, Ip = iprodione, Fp = fluopyram, and Cy = cyprodinil.

Fig. 4. UpSet plots summarizing the fungicide resistance profile in *Botrytis cinerea* over the tested years **A,** 2014 and **B,** 2018. The plots show the different combinations of multiple fungicide resistance detected in 2014 and 2018. In each panel, the bottom bar labeled with set size represents the total number of the representation of each fungicide in the resistance profile. Connected black circles in each plot represent a combination of fungicides to which one or more isolates is resistant. The top bar graph on the panels labeled as intersection size represent the number of isolates resistant to a single fungicide or a set of fungicides. The last column on the top bar graph represents the number of sensitive isolates to all eight tested fungicides detected in both years of the study.

isolates detected in our *B. cinerea* collection, in addition to all sensitive isolates detected in the same fields. Sequence alignment revealed the presence of three different point mutations in 43 isolates at amino acid position 412 from TTC to TCC, ATC, or GTC where phenylalanine (F) was replaced with serine (S), isoleucine (I), or valine (V), respectively. The frequency of detected alleles showed the prevalence of mutation F412S, with 59.2% in the Michigan *B. cinerea* populations, whereas 24.5 and 4% were detected for mutations F412I and F412V, respectively. One strain with the F412V mutation accumulated two other mutations: a replacement of asparagine by aspartic acid at amino acid position 369 with a change from AAC to GAC (N369D) (Fillinger et al. 2008) and a replacement of proline with serine at amino acid position 238 with a change from CCT to TCT (P238S) (Albertini and Leroux 2004; Avenot et al. 2020; Esterio et al. 2011; Fillinger et al. 2008). A single resistant isolate collected from a Northwest Michigan vineyard in 2018 was detected to harbor the T63I mutation (Grabke et al. 2013) where isoleucine (ACA) was substituted by threonine (ATA) at amino acid position 63. Five isolates that were phenotyped as resistant to fenhexamid and five isolates that were phenotyped as sensitive harbored both point mutations N369D and P238S. Two sensitive isolates harbored the N369D mutation and another two sensitive isolates contained the P238S mutation.

Fitness of fenhexamid resistance. At 20°C, mycelial growth rates of isolates with the F412V mutation were significantly different ($P = 0.0001$) from wild sensitive isolates, F412I, and F412S and similar results were observed at 28°C ($P = 0.00002$) (Fig. 5). At 10°C, only isolates with the F412I mutation were significantly different ($P = 0.03$) from wild sensitive isolates that grew faster; however, they were not different from the rest of the genotypes (Fig. 5). The spore germination assay at different fenhexamid concentrations resulted in no difference in resistance level between isolates with different alleles F412S/I/V, where they all remained highly resistant at 50 ppm (Supplementary Table S2). Spores of all isolates were able to germinate at 0.1 ppm, but sensitive isolates with no mutations and

isolates with mutations P238S ($n = 2$), N369D ($n = 2$), or both N369D+P238S ($n = 5$) were sensitive at 0.5 ppm and their spores did not germinate completely (Supplementary Table S2). Five isolates carrying the two point mutations N369D+P238S were resistant at all tested fenhexamid concentrations (Supplementary Table S2).

A molecular assay to detect fenhexamid resistance in *B. cinerea*. We developed a multiplex TaqMan assay to quickly genotype fenhexamid resistance in *B. cinerea* populations. The developed assay was highly specific and able to differentiate between the four alleles F/S/I/V at amino acid position 412 in *erg27* (Supplementary Fig. S2). We determined that 29, 12, and two resistant isolates had F412S, F412I, and F412V, respectively, indicating the allelic specificity of the TaqMan assay in discriminating between different alleles at the same amino acid position. Isolates with the sensitive phenotype were confirmed to have the wild allele F. The negative control did not cross-react in the assay; however, HEX or FAM may occasionally amplify in the negative control after cycle 40.

Sensitivity of the multiplex TaqMan assay was examined for individual genotypes using DNA extracted from pure culture at concentrations of 5, 1, 0.5, 0.1, 0.05, 0.001, and 0.0001 ng and a standard curve was performed. In this reaction, the four dual-labeled probes were still added as a multiplex assay for individual genotypes at the tested DNA concentrations. The TaqMan assay was highly sensitive in detecting the wild and the three mutant genotypes at all tested DNA concentrations (Fig. 6). The efficiency of the multiplex TaqMan assay for detecting the four genotypes at the tested DNA concentrations was 80.3, 73.7, 73.8, and 81.7% for the wild, S, I, and V alleles, respectively.

A total of 75 environmental samples (25 samples $\times$ 3 replicates) were tested for *B. cinerea* fenhexamid resistance detection using the *erg27* allele discrimination TaqMan test developed in this study. *B. cinerea* was detected in 65 tested samples and the TaqMan assay was successful in genotyping the detected *B. cinerea*. Detected genotypes were all sensitive to fenhexamid with wild allele F at amino acid 412, except for one Southwest Michigan vineyard that had a

Fig. 5. Temperature effect on the mycelial growth rate of *Botrytis cinerea* isolates with different mutations in the *erg27* gene in relation to fenhexamid resistance. Different letters on the bars represent significant differences between the average mycelial growth of isolates with different mutations at the tested temperatures (10, 20, and 28°C). Tukey's honest significant difference test was used ($P < 0.05$; $n = 18, 29, 12, 2, 1$, and 5 for wild, F412S, F412I, F412V, T63I, and N369D+P238S, respectively, with three replicates for each tested isolate).

resistant S allele with a cycle quantification (Cq) value of 37, while the other fluorophores had no amplification Cq values >42. Additionally, the TaqMan assay was effective in genotyping two mixed samples in two different Michigan vineyards where samples contained a resistant S allele and a sensitive allele F, indicating the high allele specificity of the reaction. These samples with mixed alleles were recovered from berries with healthy peel tissue ($n = 2$) and symptomatic nonsporulated peel tissue ($n = 2$). In these samples, the Cq values were between 30 and 35 and 35 and 39 for the F and S alleles, respectively. In general, the Cq values for samples genotyped with the F allele and extracted from sporulated, symptomatic nonsporulated, and healthy tissues were between 20 and 25, 30 and 35, and 35 and 39, respectively. The Cq values for California leaf samples genotyped with the F allele were between 23 and 36. Of the 75 samples, 10 had no amplification detected in the TaqMan test. These 10 samples included three from California leaf samples of two locations, five samples of healthy berry tissue from three Michigan locations, and two samples of symptomatic nonsporulated berry tissue of two cultivars collected in the Southwest Michigan location.

Two of five vineyards where environmental samples were collected in 2019 were the same vineyards where *B. cinerea* isolates were collected in 2014 and 2018. With the TaqMan assay using DNA extracted from environmental samples, one vineyard that had no fenhexamid resistance detected in 2014 or 2018 was confirmed for sensitivity again in 2019, while the other vineyard that had resistance detected in 2014 but not in 2018 confirmed the presence of resistance in 2019 environmental samples. The consistency of the results between the 2014, 2018, and 2019 surveys confirms the reliability of the TaqMan assay as a diagnostic tool for fenhexamid resistance detection. Successfully, the TaqMan assay was specific in detecting *B. cinerea* in mixed samples and was not influenced by the presence of grape DNA from fruit or leaves. Furthermore, we validated the TaqMan assay specificity to *B. cinerea* using the same conditions on *S. sclerotiorum* DNA, which resulted in no DNA amplification signal.

Discussion

In this study, we documented widespread fungicide sensitivity and an increasing presence of MFR in *B. cinerea* populations in Michigan vineyards. Isolates of *B. cinerea* were sampled from different grape hybrid cultivars, including Aurora, Vignoles, and Chardonnay, in addition to other *V. vinifera* cultivars, including Pinot Noir, Riesling, and Chancellor. To control gray mold/bunch rot, multiple botryticides were commonly used in these vineyards and may pose selection pressure for the development of fungicide sensitivity. Depending on the disease pressure in specific locations, a grape grower may annually apply between one to four applications specifically for *Botrytis* control

(Wise et al. 2019). We also observed the presence of fungicide sensitivity in vineyards where these fungicides were not commonly used. This is likely attributable to the spread of resistant isolates from neighboring vineyards where fungicides were commonly used, since the pathogen is able to spread by wind (Holz et al. 2007).

Resistance to multiple fungicides with different modes of action has been detected in *B. cinerea* populations in previous studies in grapes and multiple commercial crops (Amiri et al. 2013; Chen et al. 2016; Fernández-Ortuño et al. 2015; Saito and Xiao 2018; Saito et al. 2016, 2019). On table grapes in California, *B. cinerea* isolates were resistant to boscalid, cyprodinil, fenhexamid, and pyraclostrobin, and multiple resistance to the four modes of action was also detected (Saito et al. 2019). Resistance to multiple fungicides was also found in *B. cinerea* populations in German vineyards (Leroch et al. 2011; Rupp et al. 2017) and in Italy (De Miccolis Angelini et al. 2014; Panebianco et al. 2015).

In this study, high frequencies of MFR were detected in Michigan vineyards with resistance to a higher number of fungicides in 2018, indicating an increase in the risk of MFR development and challenges in controlling gray mold. Strains with MFR to six fungicides, including all tested fungicides except fluopyram and fludioxonil, dominated in 2018. We found an increase in resistance to fungicides in Northwest more than Southwest Michigan, but this might be attributable to our biased sampling in 2018, as we sampled from more locations in the northwest. However, in the same locations between the two years, development of resistance was observed in 2018 in the northwest vineyard to certain fungicides (fenhexamid and fluopyram), suggesting that use of these fungicides was repeated over the years and probably high numbers of applications were practiced. We noticed a decrease in resistance to certain fungicides in some of the locations, suggesting that with no use or proper use of these fungicides in the management of gray mold, sensitive isolates may increase in frequency in the population.

Resistance to thiabendazole, which is attributed to genetic mutation in the beta-tubulin gene (Davidse and Ishii 1995), was detected at high levels in our *B. cinerea* population, with a greater increase in 2018. This finding suggests that there are few fitness costs associated with resistance to thiabendazole as resistant strains were prevalent over the years. Our results are in agreement with results detected in mandarin in California where resistance to thiabendazole accumulated over the years (Saito and Xiao 2018). In addition, resistance to thiabendazole in *B. cinerea* was detected in apple orchards in Washington (Zhao et al. 2010) and in greenhouse vegetables in China (Zhang et al. 2010).

Resistance to pyraclostrobin, which results from mutations in the *cytb* gene, had built up over the years in the Michigan vineyards screened to reach high levels of >99% among the population, which

Fig. 6. Standard curve showing the amplification (threshold cycle [Ct]) of the *erg27* gene of *Botrytis cinerea* at different DNA concentrations ranging from 5 to 0.0001 ng specific to detect the different alleles at amino acid position 412 for **A**, wild allele, **B**, mutant F412S, **C**, mutant F412I, and **D**, mutant F412V. The linear regression equation, R^2 , and efficiency (E) of the assay are shown on the graphs.

is the highest percentage among all tested fungicides. Similar trends of a high level of resistance to pyraclostrobin were found in table grapes in California (Saito et al. 2019), in blueberry in California and Washington (Saito et al. 2016), and in strawberry and blackberry from different states (Fernández-Ortuño et al. 2015). Fitness cost to pyraclostrobin resistance was not detected in comparison with sensitive isolates (Veloukas et al. 2014). It is critical to mention that in this study, we detected a 100% level of resistance to pyraclostrobin in powdery mildew collected from six Southwest and three Northwest Michigan vineyards, while only two northwest vineyards had sensitivity/resistance or no powdery mildew detected.

Resistance to cyprodinil was confirmed in this study to increase over the years in the population. Cyprodinil resistance has been reported in the United States in various crops at high levels (Fernández-Ortuño et al. 2013b, 2015; Saito et al. 2016, 2019). Resistance to cyprodinil was reported in the central valley of California (>20%) in blueberries and grapes (Saito et al. 2016, 2019) and in strawberries in the Carolinas (Fernández-Ortuño et al. 2013b) and Florida (>45 and >50%, respectively) (Amiri et al. 2013). Cyprodinil is usually marketed in a mixture with fludioxonil as Switch for gray mold control. Although a high level of resistance to cyprodinil was detected in our study, there was very little evidence of fludioxonil resistance buildup, as we only found one resistant isolate among 240 *B. cinerea* isolates. Similarly, resistance to fludioxonil was detected on soft fruits in many different states in the United States (Fernández-Ortuño et al. 2013a, 2014a, b; Li et al. 2014). A low level of resistance to fludioxonil was also detected in grape in California (DeLong et al. 2020). Fludioxonil was the most effective fungicide in the current study and in previous studies (Amiri et al. 2013; DeLong et al. 2020; Li et al. 2014; Liu et al. 2017; Saito et al. 2016, 2019); however, with the development of *B. cinerea* isolates with resistance to different classes of fungicides with different modes of action, resistance to fludioxonil can still be a risk. With limited applications of fludioxonil and monitoring for fludioxonil resistance in *B. cinerea* in the field, the risk of developing or increasing resistance to fludioxonil may be maintained at low levels.

Resistance to SDHIs was reported previously in *B. cinerea* and other pathogens (Avenot et al. 2008; Broomfield and Hargreaves 1992; Fernández-Ortuño et al. 2012; Veloukas et al. 2014). Boscalid and fluopyram belong to SDHIs but have different chemical structure properties that make them bind differently to the target protein, succinate dehydrogenase (SDH). Therefore, cross-resistance between boscalid and fluopyram was not hypothesized and also was not detected previously in several pathosystems (Avenot et al. 2008; Fraaije et al. 2012; Ishii et al. 2011). However, several studies reported that certain mutations can confer cross-resistance between boscalid and fluopyram (Amiri et al. 2014; Esterio et al. 2015; Fernández-Ortuño et al. 2012, 2017; Leroux et al. 2010; Stammler et al. 2008; Veloukas et al. 2013, 2014). These mutations occur in subunit B of the SDH gene of *B. cinerea* and confer resistance to different fungicides within the SDHIs (Amiri et al. 2014; Fernández-Ortuño et al. 2017; Hu et al. 2016; Leroux et al. 2010; Veloukas et al. 2011; Yin et al. 2011). In our study, some isolates were resistant to boscalid only and others were resistant to both boscalid and fluopyram, suggesting the presence of such mutations in our *B. cinerea* collection.

In this study, frequencies of resistance to boscalid and pyraclostrobin were the highest in our *B. cinerea* collection. A similar trend was also observed in grapes in California (DeLong et al. 2020; Saito et al. 2019). Reduced fitness in isolates with dual resistance to QoIs and SDHIs over dual sensitive isolates has been observed (Bardas et al. 2010; Veloukas et al. 2014). However, when the use of both fungicides was discontinued for 5 years in kiwifruit orchards, isolates with dual resistance disappeared (Bardas et al. 2010; Veloukas et al. 2014). This finding suggests that dual sensitive isolates could regain dominance from dual resistant isolates in populations if these two fungicides are not being used for some years.

Resistance to fenhexamid has been observed in several U.S. states. The frequency of fenhexamid resistance is 13.7% in California vineyards (Saito et al. 2019), 29.3% in California blueberry fields (Saito et al. 2016), 17% in Carolina strawberry fields (Grabke et al. 2013),

and 30% in Florida strawberry fields (Amiri and Peres 2014). In Michigan vineyards, resistance to fenhexamid increased in 4 years from 3.4 to 38.4%, which is more than double that detected in California vineyards. This increase suggests selection pressure practiced by growers in the sampled fields by the increase in the use of fenhexamid for gray mold control. In our study, *B. cinerea* isolates were found with various point mutations in the target gene *erg27*, which have reportedly conferred high (F412S/I/V) (Albertini and Leroux 2004; Fillinger et al. 2008; Grabke et al. 2013) or weak to moderate (N369D, P238S) resistance to fenhexamid (Albertini and Leroux 2004; Avenot et al. 2020; Esterio et al. 2011; Fillinger et al. 2008). Our results of allele frequency are consistent with a previous study in France, in which the S allele was prevalent (85%) and the V allele was rare (3.5%) (Fillinger et al. 2008).

The mycelial growth rate of isolates resistant to fenhexamid and four other fungicides (iprodione, thiophanate-methyl, pyraclostrobin, and boscalid) was not different from that of wild isolates when tested at 4, 22, and 30°C, except for one isolate that grew slower at 4°C (Chen et al. 2016). This result is in contrast to a previous study in which laboratory mutant isolates with F412S/I/V mutations grew slower than sensitive isolates and had variability in sclerotia and conidial production under different temperatures and under different media (Billard et al. 2012a). All mutants had slower radial growth than the wild strain when tested in three media with different levels of nutrients at three temperatures: 11, 20, and 26°C (Billard et al. 2012a).

Although there were statistically significant differences in growth rate for some fenhexamid-resistant isolates compared with that of the sensitive isolates, our results showed that overall the resistant isolates had similar mycelial growth to the sensitive isolates, which is not in agreement with the previous finding in which laboratory mutants of fenhexamid-resistant isolates of *B. cinerea* with F412S/I/V point mutations in *erg27* had fitness cost in mycelial growth compared with the sensitive isolates (Billard et al. 2012a). However, there was a great variation in mycelial growth rate within the same group, suggesting that the variability observed under the conditions tested in this study could be attributed to the difference of individual isolates rather than to a fitness cost from fenhexamid resistance.

Although N369D and P238S mutations were reported previously (Albertini and Leroux 2004; Avenot et al. 2020; Esterio et al. 2011; Fillinger et al. 2008) to be related to the weak to moderate resistance phenotype to fenhexamid, we could not consider these mutations to be related to resistance in our *B. cinerea* Michigan collection because we found these mutations in five sensitive isolates rather than the five resistant isolates. The reason for the resistance of the five isolates carrying N369D and P238S mutations although their resistance level was >50 mg of fenhexamid remains unclear. Further studies on these isolates are needed.

Our results from examining the fitness cost by measuring the radial mycelial growth at 10, 20, and 28°C for isolates having different mutations F412S/I/V, T63I and N369D+P238S mutations in *erg27* showed that at a low temperature (10°C), only F412I was more rapid in growth than the wild isolates, which is not in agreement with Billard et al. (2012a). Our results suggest that there is a fitness cost of isolates with the F412V mutation, which was statistically significantly slower than the wild isolate at 20 and 28°C; however, that might be because we only found two among 240 isolates with this mutation. According to the mutation, growth of the fenhexamid-resistant isolates in the field might be affected under different temperatures but all resistant isolates containing the different alleles F412S/I/V and the T63 mutations were able to germinate and grow aggressively at 50 mg of fenhexamid at 20°C. We could not consider the N369D or P238S mutations related to resistance phenotype found in five isolates from our collection, since these mutations were also detected in five sensitive isolates. The reason for the resistance of these five isolates although their resistance level was >50 mg of fenhexamid is still unclear. Further studies on these isolates need to be conducted.

In this study, we developed a rapid diagnostic tool for detecting *B. cinerea* isolates with high fenhexamid resistance phenotype and differentiating three mutations at codon 412 in *erg27*. The developed

assay was reliable, rapid, specific, and sensitive with a threshold cycle value of <42 for as little as 100 fg of *B. cinerea* DNA. We also confirmed that this assay was able to differentiate environmental samples with mixed alleles and was able to detect *B. cinerea* from different materials, including grape clusters and leaves. The detection assay of *B. cinerea* resistant alleles was highly specific to *B. cinerea* and was not affected by the presence of coextracted plant DNA. The assay was also tested on *S. sclerotiorum* and resulted in no amplification confirming specificity to *B. cinerea*. Regarding sensitivity, the assay showed that we were able to recover and detect the resistant allele in *B. cinerea* in symptomatic non-sporulating as well as healthy peels of grape clusters without showing any disease symptoms that may be at early stages of infection. Although we had polysaccharides and phenolic compounds in the DNA extracted from environmental samples, amplification of the TaqMan assay was still successful. It is known that polysaccharides and phenolic compounds can inhibit the enzymatic activity of the Taq polymerase (Fang et al. 1992; John 1992). However, no inhibition to the reaction occurred during this study. The TaqMan PCR assay was successfully used previously in detecting and validating the presence of plant pathogens. A TaqMan assay was developed to detect *Fusarium fujikuroi* in different rice tissues (Carneiro et al. 2017). The reaction had high specificity toward the studied species and also did not interfere with the presence of high polysaccharides in the rice samples (Carneiro et al. 2017). In addition, a TaqMan assay was developed to detect and differentiate *Phytophthora* species (Miles et al. 2017).

TaqMan assays were developed previously to detect and quantify *B. cinerea* in different hosts, including grapes (Diguta et al. 2010; Suarez et al. 2005). However, the aim of our assay was to differentiate the mutant alleles that were reported earlier to be associated with the strong resistance profile of *B. cinerea* to fenhexamid (Albertini and Leroux 2004; Fillinger et al. 2008; Grabke et al. 2013). Because we did not find the F412C allele in our *B. cinerea* collection, we developed an in silico probe sequence to detect this allele as well as another in silico set of primers and probe for detecting the T63I mutation. Billard et al. (2012b) developed an allele-specific probe and primer amplification method to quantify the three mutations of *erg27* F412S/I/V; however, their method runs four independent assays to detect and differentiate the wild allele from the mutants. Grabke et al. (2013) developed two allele-specific PCR assays to detect the *erg27* mutation F412S/I/C and T63I; however, their assay requires running agarose gel afterward to see the results, and multiplexing in the reaction was not possible because of unspecified results. The TaqMan developed in our study is simpler and more rapid than previously developed methods. The assay developed in our study is useful in monitoring the spread of variable *B. cinerea* genotypes in Michigan vineyards.

In conclusion, this study is the first report of the prevalence of *B. cinerea* fungicide sensitivity in Michigan vineyards. We reported MFR phenotypes with resistance to fungicides with different chemical classes. Growers are advised to rotate fungicides with different modes of action and to be cautious when using fungicides with high levels of resistance, such as the premixture of pyraclostrobin and boscalid. Effective management strategies and proper use of fungicides throughout the growing season would keep gray mold resistance development at low levels in the field. Monitoring the spread of fungicide sensitivity in Michigan vineyards, identifying the resistant genotypes, and defining the mechanism of resistance will help in developing new effective fungicides for maintaining control of gray mold. In addition, the diagnostic tool developed in this study is a useful method that diagnostic clinics can use to help grape growers by recommending the best management schemes for gray mold.

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