Characterization of Postharvest Fungicide-Resistant *Botrytis cinerea* Isolates From Commercially Stored Apple Fruit

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ABSTRACT

Botrytis cinerea causes gray mold and is an economically important postharvest pathogen of fruit, vegetables, and ornamentals. Fludioxonil-sensitive *B. cinerea* isolates were collected in 2011 and 2013 from commercial storage in Pennsylvania. Eight isolates had values for effective concentrations for inhibiting 50% of mycelial growth of 0.0004 to 0.0038 µg/ml for fludioxonil and were dual resistant to pyrimethanil and thiabendazole. Resistance was generated in vitro, following exposure to a sublethal dose of fludioxonil, in seven of eight dual-resistant *B. cinerea* isolates. Three vigorously growing *B. cinerea* isolates with multiresistance to postharvest fungicides were further characterized and found to be

osmosensitive and retained resistance in the absence of selection pressure. A representative multiresistant *B. cinerea* strain caused decay on apple fruit treated with postharvest fungicides, which confirmed the in vitro results. The R632I mutation in the *Mrr1* gene, associated with fludioxonil resistance in *B. cinerea*, was not detected in multipostharvest fungicide-resistant *B. cinerea* isolates, suggesting that the fungus may be using additional mechanisms to mediate resistance. Results from this study show for the first time that *B. cinerea* with dual resistance to pyrimethanil and thiabendazole can also rapidly develop resistance to fludioxonil, which may pose control challenges in the packinghouse environment and during long-term storage.

According to the U.S. Apple Association, the farm gate value of the U.S. apple crop in 2014 was valued at \$2.7 billion. Apple fruit are handpicked into bags and placed directly into wooden or plastic bins in the field for transport to the storage or packing facilities. Fruit are typically stored for up to 6 months at 1°C in air and can be kept for 1 year in controlled-atmosphere storage, where temperature, oxygen, and carbon dioxide are maintained at specific levels (Robinson et al. 2014). Extended storage times provide consumers with high-quality fruit year round and give the producer the opportunity to sell based on market demand. However, as fruit mature, their susceptibility to various fungal diseases increases, resulting in escalating economic losses (Xiao and Boal 2009).

Botrytis cinerea Pers. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) causes gray mold of apple fruit during storage and is a major pathogen of apple worldwide (Xiao 2014). Kim and Xiao (2008) reported that gray mold accounted for 28% of the decayed apple fruit in commercial storage in Washington State. The fungus has an extensive host range exceeding 200 plant species, including ornamentals, vegetables, and many types of fruit pre- and post-harvest (Jarvis 1977). Infection primarily occurs via wounds and other surface defects that occur during and after harvest and in storage, creating infection courts that manifest in decay (Zhao et al. 2010). Control of this disease is achieved via fungicide application and cultural practices (Xiao and Boal 2009). However, the fungus remains a threat to the pome fruit industry due to its ability to reproduce rapidly and survive adverse conditions for extended periods of time, and its potential to mutate at high frequencies to overcome fungicide treatments (Fillinger et al. 2012).

There are four postharvest fungicides currently registered in the United States to control gray mold caused by *Botrytis cinerea* on apple fruit: fludioxonil (Scholar 230SC; Syngenta Crop Protection), fludioxonil + difenoconazole (Academy; Syngenta Crop Protection), pyrimethanil (Penbotec 400SC; Janssen Pharmaceutica N.V.), and thiabendazole (Mertect 340F; (Syngenta Crop Protection). Application of these products can vary according to the label but, in general, they are applied as drenches prior to storage, as dips or line sprays or in waxes during the sorting and packing process prior to storage, or thermo-fogged in storage rooms. Thiabendazole (TBZ) inhibits β -tubulin synthesis and was labeled for blue mold control in 1968 (Zhao et al. 2010). Both fludioxonil (FLU) and pyrimethanil (PYR) were registered for postharvest use on apple fruit in 2004 to control blue mold (*Penicillium* spp.) and gray mold (Xiao and Boal 2009). FLU inhibits the osmosensing pathway, thereby preventing conidial germination and mycelial growth (Li and Xiao 2008). PYR has preventative and curative effects and blocks spore germination, germ tube elongation, and secretion of fungal cell-wall-degradation enzymes (Kanetis et al. 2008). In 2016, a premix postharvest fungicide, Academy, was introduced to the market and contains FLU and difenoconazole (DIF), which targets the fungal osmosensing and sterol biosynthesis pathways.

The bulk of studies on resistance to FLU in *B. cinerea* have focused on naturally occurring isolates obtained from the field or packinghouse with histories of the fungicide use (Kretschmer et al. 2009; Li et al. 2014; Zhao et al. 2010). One naturally occurring *B. cinerea* isolate from apple, with reduced sensitivity to FLU, was osmosensitive, less virulent on apple fruit, and stable in the absence of fungicide selection pressure (Zhao et al. 2010). FLU-resistant *B. cinerea* isolates from blackberry were virulent on strawberry fruit, osmosensitive, and consistently associated with the arginine to isoleucine (R632I) mutation in the *Mrr1* gene (Li et al. 2014). Currently, there is no information concerning the potential of *B. cinerea* strains with dual resistance against two different classes of postharvest fungicides to develop resistance to FLU. This

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scenario has a strong potential to occur in the controlled environment of the packing and storage facilities following exposure to different postharvest fungicides with varying modes of action. Development of multiple postharvest fungicide-resistant strains could have a devastating effect on the pome fruit packing industry and research is needed to address fundamental questions of the fungus's ability to develop resistance, capacity to retain acquired resistance, and decay-causing potential on postharvest fungicide-treated fruit. Therefore, the objectives of this investigation were to (i) determine the potential of dual-postharvest fungicide (PYR/TBZ)-resistant *B. cinerea* isolates to develop resistance to FLU in vitro; (ii) characterize in vitro growth, osmotic sensitivity, and retention of a multipostharvest fungicide resistance phenotype of *B. cinerea* isolates; (iii) determine the efficacy of three postharvest fungicides registered for pome fruit to control gray mold caused by a multifungicide-resistant *B. cinerea* strain; and (iv) examine the C-terminal portion of the *Mrr1* transcription factor in *B. cinerea* isolates to investigate the potential mechanisms of resistance.

MATERIALS AND METHODS

Fungal isolates, fungicides, and fruit. In total, 110 *B. cinerea* isolates were obtained from decayed apple fruit with gray mold symptoms from a commercial storage facility with a history of postharvest fungicide use located in Pennsylvania in 2011 and 2013. These isolates were obtained from decayed 'Fuji' and 'Gala' apple fruit with gray mold symptoms. Eight of these isolates were selected based on their sensitivity to FLU and resistance to TBZ and PYR, high rate of sporulation, and vigorous growth in culture. These eight isolates were started from single-spore cultures and maintained on potato dextrose agar (PDA) agar at 4°C. Technical-grade stocks of FLU, TBZ, and PYR were obtained from Sigma-Aldrich and used for all in vitro tests. Concentrated stock solutions of 10,000 µg/ml were prepared by dissolving them in appropriate solvents for FLU (acetone), PYR (acetone), and TBZ (dimethyl sulfoxide) and kept in the dark at 4°C. Commercial formulations of these postharvest fungicides—Scholar 230SC (Syngenta Crop Protection), Penbotec 400SC (Janssen Pharmaceutica N.V.), and Mertect 340F (Syngenta Crop Protection)—were used in the apple decay tests. 'Golden Delicious' apple fruit were harvested at the Penn State Fruit Research and Extension Center in Biglerville, PA at commercial maturity, with average starch scores of 5 on an 8-point scale. No preharvest fungicides with postharvest efficacy or postharvest fungicides were applied to these apple fruit and they were stored on commercial tray packs in cardboard apple boxes at 1°C in air.

Fungicide phenotyping and determination of effective concentrations for inhibiting 50% of mycelial growth. The fungicide phenotype for eight *B. cinerea* isolates was determined using discriminatory doses previously determined by Zhao et al. (2010). Briefly, 4.5-cm petri dishes containing PDA amended with FLU (0.5 µg/ml) or TBZ (10 µg/ml) and Richard's defined medium amended with PYR (1.0 µg/ml) were inoculated with 4-mm mycelial plugs taken from the margin of a 7-day-old *B. cinerea* colony actively growing on PDA. There were three replicates per treatment. An isolate was considered resistant if new mycelial growth was observed 3 days postinoculation onto the amended medium. Effective concentrations for inhibiting 50% of mycelial growth (EC₅₀) were determined for FLU using 0, 0 + carrier, 0.0001, 0.001, 0.01, 0.1, and 1.0 µg/ml for FLU-sensitive dual-resistant isolates and 0, 0 + carrier, 0.01, 0.1, 1.0, 10, and 50 µg/ml for multiple-resistant *B. cinerea* strains with conditioned resistance to FLU. Mycelial plugs (6.25 mm in diameter) of *B. cinerea*, were transferred onto the PDA with FLU, and incubated for 3 days at 20°C with 12-h cycles of light and darkness. Colony diameter was recorded for each concentration using a digital micrometer.

Generating FLU resistance in vitro. Eight dual-resistant *B. cinerea* isolates were grown on PDA with the lowest dose of FLU (0.065 µg/ml) that would suppress mycelial growth in culture. After

growth on the sublethal dose, three hyphal plugs from the margin of actively growing colonies were transferred to PDA plates with different concentrations of FLU (0.1, 0.25, and 0.5 µg/ml) in triplicate. The number of plates on which the fungus grew containing fungicide-amended medium was recorded and expressed as the percentage of the total number of plates inoculated for a given concentration (percent growth). Five days postinoculation, isolates that grew on FLU-PDA at 0.5 µg/ml were transferred to 0.5-µg/ml FLU-PDA plates, and this was repeated. Three vigorously growing isolates on the discriminatory dose of FLU at 0.5 µg/ml were designated -C (for conditioned) and selected for further characterization. One isolate, 3RGA27, did not grow directly on FLU-PDA at 0.5 µg/ml postconditioning. Therefore, it was propagated in stepwise fashion on FLU-PDA at 0.1, 0.25, and 0.5 µg/ml. The isolate was capable of growing on FLU-PDA at 0.5 µg/ml after a stepwise process but died upon subsequent transfer to FLU-PDA at 0.5 µg/ml. All treatments contained three replicates.

Polymerase chain reaction and phylogenetic analysis.

Genomic DNA was extracted from fungal cultures grown on PDA colonized plates. Fungal mycelium, with the agar removed, was extracted with the Sigma Extract-N-Amp kit to obtain genomic DNA. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and *Mrr1* transcription factor genes were amplified using conventional polymerase chain reaction (PCR) and primers designed according to Li et al. (2014) and Grant-Downton et al. (2014) (Table 1). Each amplicon was sequenced using the Sanger method at the University of Florida Interdisciplinary Center for Biotechnology Research sequencing lab and a 2× consensus was subjected to a MegaBLAST analysis for species-level identification. The partial *Mrr1* gene was translated using in silico tools and compared with other *Mrr1* peptide sequences obtained from GenBank. Multiple sequence alignment was achieved using ClustalW and residue 632 was examined for the nonsynonymous mutation (R to I) that has been reported to coincide with resistance to FLU. *B. cinerea* nucleotide sequences were obtained from GenBank associated with published sources and aligned using MUSCLE. A phylogenetic tree was constructed using maximum-likelihood parsimony (unweighted pair-group method with arithmetic means, Jukes-Cantor model) in the CLC Genomics Workbench, version 7.5.1. Bootstrap analysis was performed with a 70% threshold. Both *Sclerotinia sclerotiorum* and *B. allii* were included in the analysis as outgroups.

In vitro growth rate and stability of fungicide resistance.

B. cinerea isolates were grown on five types of media: tryptic soy agar (TSA), malt extract agar (MEA), V8 agar (V8), PDA, and PDA with 4% NaCl. Conditioned isolates were grown on PDA and PDA containing 4% NaCl. Plates were incubated at 20°C with 12-h cycles of light and darkness, and colony diameters were measured using a digital micrometer. To evaluate the stability of FLU, PYR, and TBZ resistance, three conditioned isolates were cultured for 10 generations on PDA without selection in triplicate. Following the 10th generation, each isolate was transferred to either PDA containing FLU at 0.5 µg/ml or TBZ at 10 µg/ml and Richard's defined medium containing PYR at 1.0 µg/ml and assessed for growth. Each isolate was tested in triplicate and the experiments were repeated.

Efficacy of postharvest fungicides to control gray mold on apple fruit. Conditioned multiresistant isolate B9F-C (resistant to FLU, PYR, and TBZ) and parental dual-resistant isolate B9F-P (sensitive to FLU, resistant to PYR and TBZ) were selected

TABLE 1. Primer names, sequences, and resulting amplicon sizes used for characterization of *Botrytis cinerea* isolates

Primer name	Sequence 5'–3'	Amplicon size (bp)
BcMrr1-F	tgcgtacagctatggcattc	631
BcMrr1-R	ccccacatctctggattttg	...
Bc-G3PDH-F	gatggattggagggtcaatgg	728
Bc-G3PDH-R	gtcaccggttcattgtcagttg	...

for further evaluation based on their ability to grow vigorously and sporulate well in culture. Apple fruit were washed with soap and water and dried with sterile paper towels, then sanitized by spraying with 70% ethanol to runoff. The fruit were dried once more with paper towels and placed on 20-count cardboard trays. Apple fruit were wounded once at the equator with the point of a finishing nail to a depth of 3 mm and wound inoculated with 20 µl of a *B. cinerea* conidial suspension adjusted to 1×10^4 conidia/ml. One hour after inoculation, fruit were dipped for 30 s with constant agitation in either deionized water or one of the three fungicide solutions of Scholar 230SC (0.94 ml/liter), Mertect 340F (1.25 ml/liter), or Penbotec 400SC (1.25 ml/liter) at rates indicated on the fungicide labels (Zhao et al. 2010). After dip treatment, the fruit were air dried for 30 min, placed on trays, and stored in cardboard apple boxes for 7 days at 22°C in the dark. Lesion diameters originating from each wound were measured. The incidence of decay and disease severity were calculated. Twenty fruit were used for each treatment and the experiment was repeated.

Statistical analysis. All experiments were performed at least twice unless indicated otherwise. Data from independent experiments were pooled for analysis only if they were not deemed statistically different ($P > 0.05$) via an *F* test for equality of variances. Linear regression of percent inhibition of mycelial growth relative to the control versus logarithmic transformation for each concentration of FLU was carried out using SAS PROC REG (version 9.1; SAS Institute) and the EC₅₀ values were calculated. All percentage data were arcsine-square-root transformed prior to analysis so as not to violate analysis of variance assumptions. Mycelial growth rate, percent survival, and decay analysis of apple fruit were analyzed using analysis of variance (PROC GLM) in SAS, and means were separated by the Waller-Duncan *K*-ratio *t* test, where *K* = 100.

RESULTS

Characterization of *B. cinerea* isolates. Growth on different media and EC₅₀ of eight *B. cinerea* isolates are shown in Table 2. All isolates were determined to be sensitive to FLU but were dual resistant to PYR and TBZ using discriminatory doses of 0.5, 1.0, and 10 µg/ml, similarly to those previously reported by Zhao et al. (2010). For FLU, the EC₅₀ was 0.0004 to 0.0038 µg/ml. Mycelial growth was determined on three separate types of media and the isolates grew best on MEA, with growth of 4.89 to 7.15 mm/day, and on V8, with 4.44 to 7.18 mm/day, and significantly less on TSA, with growth of 1.66 to 4.08 mm/day (Table 2).

Multiple sequence alignment of the C-terminal region of the *Mrr1* locus revealed the presence of an arginine residue at position 632 in all parental isolates, including those obtained from GenBank for comparison that were FLU sensitive (MC14) and a *B. cinerea*

(CB82a) strain from blackberry with low resistance to FLU (Fig. 1A). Isolate KC33 was moderately resistant to FLU (capable of growth on FLU at 10 µg/ml) and was included to show the isoleucine residue at position 632 that coincides with FLU resistance. Three representative *B. cinerea* isolates with conditioned resistance to FLU (B9F, 3RGA36, and 3RGA39) growing with FLU at 0.5, 1.0 and 10 µg/ml were analyzed for the R631I mutation alongside three isolates from blackberry (sequences obtained from GenBank) that were sensitive, low resistant, and moderately resistant to FLU. Unexpectedly, arginine was found at position 632 in all isolates capable of growing on three different concentrations of FLU (Fig. 1B).

BLAST analysis of 2× consensus for the G3PDH gene matched 100% with other *B. cinerea* isolates in the GenBank and confirmed the species-level identification. These sequences were also used to assess phylogenetic relationships of the *B. cinerea* isolates from Pennsylvania and other geographic locations (Fig. 2). Phylogenetic analysis using the G3PDH locus showed that the isolates from Pennsylvania composed a distinct clade, with 100% bootstrap support, differing from isolates from South Carolina, China, and The Netherlands. Sequence data from *S. sclerotiorum* 1980 and *B. allii* MUCL43 were included as outgroups.

Generating conditioned resistance to FLU. Growth on a sublethal dose of FLU (0.065 µg/ml) followed by transfer to either 0.1, 0.25 or 0.5 µg/ml resulted in all isolates growing at the lowest concentration of FLU; however, apparent variation in growth was observed among the isolates challenged at the higher concentrations (Table 2). Interestingly, one isolate (3RGA27) did not grow with FLU at 0.25 or 0.5 µg/ml postconditioning (Table 2). Therefore, to determine whether this isolate could reach the discriminatory dose of FLU at 0.5 µg/ml, it was transferred in a stepwise fashion from 0.1 to 0.25 and then 0.5 µg/ml (data not shown). This isolate did not grow upon subsequent transfer on PDA amended with a discriminatory dose of FLU (Table 3). Isolates B9F, 3RGA36, and 3RGA39 grew vigorously on PDA amended with FLU at 0.5 µg/ml; they were designated -C and selected for further characterization.

Three multiple-resistant *B. cinerea* isolates (B9F-C, 3RGA36-C, and 3RGA39-C) with conditioned resistance to FLU were analyzed for EC₅₀, resistance to PYR and TBZ, growth rate on PDA and PDA amended with 4% NaCl, and stability of resistance and compared with their parental FLU-sensitive strains (Table 4). EC₅₀ for FLU in conditioned isolates was >50 µg/ml for all three strains, which is several orders of magnitude higher than their respective parental isolates, which ranged from 0.0021 to 0.0023 µg/ml. Development of FLU resistance did not affect dual resistance to PYR and TBZ and was determined to be stable after 10 successive generations in the absence of selection pressure (Table 4). The growth rate of conditioned isolates was approximately half that of the parental

TABLE 2. Characterization of *Botrytis cinerea* isolates from Pennsylvania for fungicide resistance and growth rates on different media

Isolate ^w	Cultivar ^x	EC ₅₀ (µg/ml) ^y	Fungicide phenotype ^z	Mycelial growth (mm/day) ^v		
				TSA	MEA	V8
B9F	Fuji	0.0021	FLU _S PYR _R TBZ _R	2.54 b	6.35 a	5.67 a
3RGA9	Gala	0.0024	FLU _S PYR _R TBZ _R	3.00 b	4.89 a	4.97 a
3RGA22	Gala	0.0038	FLU _S PYR _R TBZ _R	2.76 b	6.52 a	7.18 a
3RGA27	Gala	0.0004	FLU _S PYR _R TBZ _R	1.70 b	6.00 a	4.55 a
3RGA33	Gala	0.0033	FLU _S PYR _R TBZ _R	2.81 b	6.39 a	5.75 a
3RGA36	Gala	0.0023	FLU _S PYR _R TBZ _R	1.66 b	7.15 a	5.95 a
3RGA37	Gala	0.0024	FLU _S PYR _R TBZ _R	4.08 b	6.39 a	6.65 a
3RGA39	Gala	0.0021	FLU _S PYR _R TBZ _R	3.50 b	6.02 a	5.95 a

^v Mycelial growth rate expressed in millimeters per day on tryptic soy agar (TSA), malt extract agar (MEA), and V8 agar (V8) at 25°C with natural light. Numbers within the same row for a given medium followed by different letters are statistically significant according to the Waller-Duncan *K*-ratio *t* test, where *K* = 100.

^w All cultures are maintained at United States Department of Agriculture–Agricultural Research Service Food Quality Laboratory in Beltsville, MD.

^x *B. cinerea* was isolated from decayed apple fruit from a commercial storage facility in Pennsylvania and propagated as monoconidial cultures.

^y Mean values for effective concentrations for inhibiting 50% of mycelial growth (EC₅₀) for *B. cinerea* isolates were determined using potato dextrose agar (PDA) amended with technical-grade fludioxonil and calculated using linear regression models in SAS.

^z *B. cinerea* isolates were considered resistant (R) if they grew or sensitive (S) if they did not grow on discriminatory doses of PDA amended with technical-grade fludioxonil (FLU) at 0.5 µg/ml, Richard's defined medium amended with pyrimethanil (PYR) at 1 µg/ml, or PDA with thiabendazole (TBZ) at 10 µg/ml.

A

B9F-P	ELCAEPTGPLADQAWEGIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
3RGA9B-P	ELCAEPTGPLADQAWEGIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
3RGA22B-P	ELCAEPTGPLADQAWEGIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
3RGA27B-P	ELCAEPTGPLADQAWEGIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
3RGA33B-P	ELCAEPTGPLADQAWEGIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
3RGA36B-P	ELCAEPTGPLADQAWEGIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
3RGA37B-P	ELCAEPTGPLADQAWEGIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
3RGA39B-P	ELCAEPTGPLADQAWEGIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
MC14-S (0)	ELCAEPTGPLADQAWEGIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
CB82a-LR (1.0)	ELCAEPTGPLADQAWGSIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
KC33-MR (10.0)	ELCAEPTGPLADQAWGSIEFHYNKWSEFVADTKDGMWIRPVNLMKARAARRRERGLSE

B

B9F-C (0.5)	ELCAEPTGPLADQAWEGIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
B9F-C (1.0)	ELCAEPTGPLADQAWEGIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
B9F-C (10.0)	ELCAEPTGPLADQAWEGIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
3RGA36B-C (0.5)	ELCAEPTGPLADQAWEGIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
3RGA36B-C (1.0)	ELCAEPTGPLADQAWEGIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
3RGA36B-C (10.0)	ELCAEPTGPLADQAWEGIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
3RGA39B-C (0.5)	ELCAEPTGPLADQAWEGIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
3RGA39B-C (1.0)	ELCAEPTGPLADQAWEGIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
3RGA39B-C (10.0)	ELCAEPTGPLADQAWEGIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
MC14-S (0)	ELCAEPTGPLADQAWEGIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
CB82a-LR (1.0)	ELCAEPTGPLADQAWGSIEFHYNKWSEFVADTKDGMWIRPVNLMKRARAARRRERGLSE
KC33-MR (10.0)	ELCAEPTGPLADQAWGSIEFHYNKWSEFVADTKDGMWIRPVNLMKARAARRRERGLSE

Fig. 1. Multiple amino acid sequence alignment of the *Mrr1* C-terminus using MUSCLE highlighting (shaded) the amino acid residue (R632I) associated with resistance to fludioxonil for **A**, parental (-P; dual-resistant and fludioxonil-sensitive) and **B**, conditioned (-C; multiresistant and fludioxonil-resistant) *Botrytis cinerea* isolates capable of growing on potato dextrose agar amended with fludioxonil at 0.5, 1.0, and 10 µg/ml. Additional isolates for comparison were obtained from GenBank. S = sensitive, LR = low resistant, and MR = moderately resistant to fludioxonil.

Fig. 2. Phylogenetic tree of *Botrytis cinerea* isolates using highly conserved locus glyceraldehyde-3-phosphate dehydrogenase. In addition to nucleotide sequences for Pennsylvania isolates, sequences were also obtained for *B. cinerea* from The Netherlands, China, and South Carolina and for designated outgroups *B. allii* and *Sclerotinia sclerotiorum* from GenBank. Sequences were aligned using MUSCLE, trimmed, and analyzed using maximum-likelihood phylogeny. Statistical support for each branch is indicated by bootstrap values (1,000 replicates).

strains on PDA and they were osmosensitive, with growth significantly reduced on PDA amended with 4% NaCl.

Efficacy of postharvest fungicides to control gray mold on apple. Golden Delicious apple fruit inoculated with either dual-resistant (FLU-sensitive) or multiple-resistant (FLU-resistant) *B. cinerea* isolates and treated with three different postharvest fungicides to determine their effectiveness in controlling gray mold (Table 5). Decay was observed on apple fruit inoculated with a B9F dual-resistant parental isolate treated with Mertect (TBZ) and Penbotec (PYR) but not with Scholar (FLU). Decay incidence and severity were not significantly different between untreated and Mertect-treated apple fruit but were significantly lower in Penbotec and Scholar treatments (Table 5). Decay was observed on Mertect-, Penbotec-, and Scholar-treated apple fruit when inoculated with the multiresistant B9F-C isolate that was resistant to Mertect, Penbotec, and Scholar. Decay incidence was significantly higher on Scholar and was lowest on Penbotec-treated fruit. However, mean lesion sizes of infected apple fruit were not statistically different for untreated or Mertect- and Penbotec-treated apple fruit but were significantly smaller in Scholar-treated fruit.

DISCUSSION

B. cinerea isolates from Pennsylvania had EC₅₀ values that were similar to FLU-sensitive baseline isolates from apple in the United States and comparable with those from strawberry in northeastern Switzerland (Hilber et al. 1995; Zhao et al. 2010). This indicates that the *B. cinerea* isolates investigated in the

current study have similar levels of sensitivity to FLU compared with isolates from other geographically distinct areas. The isolates were also found to have dual resistance to TBZ and PYR, which has not been demonstrated to date for *B. cinerea* from pome fruit but has been shown for laboratory-generated mutants of *Penicillium expansum* (Li and Xiao 2008). The parental isolates were obtained from a major commercial packing and storage facility in Pennsylvania with a history of postharvest fungicide use, which may explain their dual fungicide resistance phenotypes. The concept of fungicide-driven evolution to multiple fungicide classes has been shown for field populations of *B. cinerea* from small fruit in the United States and on wine grapes in Europe, which supports our findings (Fernández-Ortuño et al. 2015; Kretschmer et al. 2009). Although scientifically interesting, these results are troubling from a practical standpoint because dual- or multiple-fungicide-resistant isolates would be difficult to control in the packinghouse environment, considering that there are only four currently labeled fungicides for control of postharvest gray mold on apple. These results also reinforce the need for additional research to develop new strategies for gray mold control to maintain apple fruit quality during long-term storage.

Genetic characterization of the parental isolates was conducted using a highly conserved locus that has been implemented in *Botrytis* speciation and phylogeny studies (Grant-Downton et al.

TABLE 3. Growth of dual-resistant *Botrytis cinerea* isolates on potato dextrose agar amended with various concentrations of technical-grade fludioxonil

Isolate name	Growth (%) with fludioxonil (µg/ml) ^z			
	0.065	0.1	0.25	0.5
B9F	100	100	83	100*
3RGA9	100	100	50	33
3RGA22	100	100	67	67
3RGA27	100	100	0	0
3RGA33	100	100	33	50
3RGA36	100	100	67	33*
3RGA37	100	100	83	67
3RGA39	100	100	50	67*

^z Percent growth was calculated by dividing the number of plates with growth by the total number of mycelial plugs inoculated onto a given dose of fludioxonil-amended potato dextrose agar (PDA). A sublethal dose of fludioxonil (0.065 µg/ml) was used to condition isolates prior to subsequent transfer to higher doses of fludioxonil from 0.1 to 0.5 µg/ml. An asterisk (*) indicates vigorous mycelial growth after two transfers to PDA containing fludioxonil at 0.5 µg/ml.

TABLE 5. Control of *Botrytis cinerea* dual-resistant parental (P) or multi-resistant conditioned (C) isolates on wound-inoculated apple fruit treated with labeled rates of commercial postharvest fungicides (Scholar, Penbotec, and Mertect) stored at 20°C for 7 days

Isolate, phenotype, treatment ^y	Incidence (%)	Lesion size (mm) ^z
B9F-P, FLU_SPYR_RTBZ_R		
Untreated	100 a	59.5 a
Mertect	100 a	60.3 a
Penbotec	92.5 b	45.6 b
Scholar	0 c	0 c
B9F-C, FLU_RPYR_RTBZ_R		
Untreated	37.5 ab	24.2 a
Mertect	22.5 b	25.3 a
Penbotec	2.50 c	33.8 a
Scholar	45.0 a	7.93 b

^y FLU = fludioxonil, PYR = pyrimethanil, TBZ = thiabendazole, S = sensitive, and R = resistant. Untreated, Mertect, Penbotec, and Scholar were applied as sterile deionized water, Mertect 340F at 1.25 ml/liter, Penbotec 400SC at 1.25 ml/liter, and Scholar 230SC at 0.94 ml/liter, respectively.

^z Lesion size in millimeters represents the lesion diameter, including the wound of approximately 3 mm. Values within a column for each isolate followed by a different letter are significantly different according to the Waller-Duncan *K*-ratio *t* test, where *K* = 100 (*P* = 0.05).

TABLE 4. Fungicide sensitivity and phenotypic characteristics of dual-resistant parental and multiresistant conditioned isolates from Pennsylvania

Isolate name ^w	EC ₅₀ (µg/ml) ^x	Fungicide phenotype ^y	Growth rate (mm/day) on ^v		Phenotype ^z
			PDA	PDA + 4% NaCl	
B9F-P	0.0021	FLU _S PYR _R TBZ _R	10.4 a	9.8 b	nd
3RGA36-P	0.0023	FLU _S PYR _R TBZ _R	7.0 b	7.3 c	nd
3RGA39-P	0.0021	FLU _S PYR _R TBZ _R	7.1 b	11.2 a	nd
B9F-C	>50	FLU _R PYR _R TBZ _R	5.9 c	3.1 d	FLU _R PYR _R TBZ _R
3RGA36-C	>50	FLU _R PYR _R TBZ _R	4.4 d	3.2 e	FLU _R PYR _R TBZ _R
3RGA39-C	>50	FLU _R PYR _R TBZ _R	3.9 d	2.3 d	FLU _R PYR _R TBZ _R

^v Mycelial growth on potato dextrose agar (PDA) at 25°C expressed in millimeters per day. Numbers within the same column followed by different letters are statistically significant according to the Waller-Duncan *K*-ratio *t* test, where *K* = 100 (*P* = 0.05).

^w P = parental isolate sensitive to fludioxonil and resistant to pyrimethanil and thiabendazole, and C = conditioned multiresistant isolate capable of growing with fludioxonil at 0.5 µg/ml.

^x Mean values for effective concentrations for inhibiting 50% of mycelial growth (EC₅₀) for *Botrytis cinerea* isolates was determined using PDA amended with technical-grade fludioxonil and calculated using linear regression models in SAS.

^y *B. cinerea* isolates were considered resistant (R) if they grew or sensitive (S) if they did not grow on discriminatory doses of PDA amended with technical-grade fludioxonil (FLU) at 0.5 µg/ml, Richard's defined medium amended with pyrimethanil (PYR) at 1 µg/ml, or PDA with thiabendazole (TBZ) at 10 µg/ml.

^z Phenotype after 10 generations without selection; nd = not determined.

2014; Staats et al. 2005). MegaBLAST analysis confirms our morphological identification at the species level and rules out the possibility of a different *Botrytis* spp. that could have represented an emerging apple pathogen. It is conceivable that a new species may be found causing postharvest decay of apple in the future, similar to recent findings for strawberry (*B. caroliniana*), daylily (*B. deweyae*), blueberry (*B. californica*), and table grape (*B. sinoviticola*) (Grant-Downton et al. 2014; Li et al. 2012; Saito et al. 2016; Zhou et al. 2014). Our original hypothesis was that the *B. cinerea* isolates from Pennsylvania would be genetically similar to those from South Carolina, based on geographic proximity. However, analysis using the GAPDH locus revealed otherwise, because isolates from Pennsylvania composed a distinct clade from *B. cinerea* isolates from South Carolina, China, and The Netherlands.

This is the first investigation to demonstrate that *B. cinerea* can rapidly develop resistance to FLU in isolates that are dual resistant to postharvest fungicides following exposure to the active ingredient in Scholar. Most of the investigations concerning fungicide-resistant gray mold have been focused on naturally occurring *B. cinerea* isolates from apple, blackberry, and strawberry in the United States (Fernández-Ortuño et al. 2015; Li et al. 2014; Zhao et al. 2010). In our study, seven of the eight isolates grew on a discriminatory dose of FLU after conditioning and had EC₅₀ values greater (>50 µg/ml) than what has been reported in the literature for a naturally occurring FLU-reduced-sensitive *B. cinerea* isolate from apple (Zhao et al. 2010). Furthermore, analysis of *B. cinerea* isolates with conditioned resistance to FLU showed that they were osmosensitive and that resistance was stable in the absence of selection pressure, which is consistent with reports concerning FLU resistance in *B. cinerea* (Li et al. 2014; Zhao et al. 2010). The *B. cinerea* isolates with conditioned resistance to FLU also had reduced fitness because their growth rates were approximately 50% less on PDA compared with growth of their parental strains on the same medium. However, they still caused decay on apple fruit treated with Scholar, Penbotec, and Mertect. Despite their reduction in growth and aggressiveness, these multiresistant isolates have the potential to cause decay in the packinghouse because the currently labeled materials (with the possible exception of Academy) would be ineffective.

In *B. cinerea*, one of the genes responsible for the extracellular transport of fungicides is a homolog of the *atrB* gene from *Aspergillus nidulans* (Vermeulen et al. 2001). Resistance to FLU has been associated with a mutation in the *Mrr1* transcription factor and is designated multidrug resistance 1 (MDR1) for *B. cinerea* from grape, blackberry, and strawberry (Kretschmer et al. 2009; Li et al. 2014). Analysis of the C-terminal portion of *Mrr1* from the parental *B. cinerea* isolates investigated in this study contained the expected arginine residue at position 632, which coincided with susceptibility to FLU. However, analysis of three multiresistant *B. cinerea* isolates growing on FLU at 0.5, 1.0, and 10 µg/ml lacked the expected isoleucine residue at position 632 and, instead, showed arginine. These results indicate that the mechanisms of resistance that occur during conditioning in the laboratory may be different from what is naturally occurring in isolates from the field (Kretschmer et al. 2009; Li et al. 2014). Other common mechanisms, including sequestration, detoxification, or metabolism of FLU, may be acting in isolation or in combination to confer conditioned resistance in these isolates and is the subject of future investigations in our laboratory using omics-based approaches.

In conclusion, *B. cinerea* isolates with dual resistance to TBZ and PYR have been detected and characterized for the first time from isolates originating from a commercial packing and storage facility in the Mid-Atlantic region. Results from this study have demonstrated that isolates with dual resistance to TBZ and PYR have the potential to rapidly develop resistance to FLU, which can cause decay on postharvest fungicide-treated fruit. Our findings demonstrated that the development of

multiple resistance to postharvest fungicides in *B. cinerea* isolates is a potential threat to control of apple gray mold because such strains may be able to persist in the packinghouse in the absence of the fungicide selection pressure. As with any decay control program, integration of cultural and chemical practices is essential for optimal control and to preserve the longevity and efficacy of these chemicals. Therefore, it is sensible to rotate the use of postharvest fungicides with different active ingredients frequently, according to the Fungicide Resistance Action Committee code, based on fungicide use histories not only in the postharvest handling and storage facility but also in the field.

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