Resistance to Fludioxonil in *Botrytis cinerea* Isolates from Blackberry and Strawberry

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ABSTRACT

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Site-specific fungicides, including the phenylpyrrole fludioxonil, are frequently used for gray mold control but are at risk for the development of resistance. In this study, field isolates that were low-resistant (LR) and moderately resistant (MR) to fludioxonil from blackberry and strawberry fields of North Carolina, South Carolina, and Virginia were characterized. Genes involved in osmoregulation, including *bcsak1*, *BcOS4*, *bos5*, and *BRRG-1*, were cloned and sequenced to detect potential target gene alterations; however, none were found. A previously described mutation (R632I) in transcription factor Mrr1, which is known to increase the expression of ATP-binding cassette transporter AtrB, was found in MR but not in sensitive (S) or LR isolates. Expression of *atrB* in MR isolates

was ≈200-fold increased compared with an S isolate; however, 30- to 100-fold overexpression was also detected in LR isolates. Both MR isolates exhibited increased sensitivity to salt stress in the form of mycelial growth inhibition at 4% NaCl, indicating a disruption of osmoregulatory processes in those strains. However, the glycerol content was indistinguishable between S, LR, and MR isolates with and without exposure to fludioxonil, suggesting that the glycerol synthesis pathway may not be a part of the resistance mechanism in LR or MR strains. An investigation into the origin of LR and MR isolates from blackberry revealed two insertions in the *mrr1* gene consistent with those found in the *Botrytis* clade group S. The emergence of strains overexpressing *atrB* in European and now in North American strawberry fields underscores the importance of this resistance mechanism for development of resistance to fludioxonil in *Botrytis cinerea*.

Fludioxonil is a phenylpyrrole fungicide with broad-spectrum activity against fungal plant pathogens among ascomycetes and basidiomycetes (12). Fludioxonil is effective against most diseases of seed, including seedling blight, stem-base browning, snow mold, and common bunt. It is very effective against *Botrytis cinerea* Pers., the causal agent of gray mold disease of many vegetables and small fruits, and inhibits both mycelial growth and spore germination (24). It is registered in the United States against various post-harvest rots of pome fruits, stone fruits, and sweet potato. Fludioxonil was first registered in 2002 and is listed under Fungicide Resistance Action Committee (FRAC) code 12 with FRAC resistance risk category low to medium.

Resistance in *B. cinerea* to fludioxonil is rarely found in the field (7,25,34) worldwide. One isolate from apple grown in Washington State was moderately resistant (MR) to fludioxonil but was impaired in fitness and pathogenicity on apple fruit (43). Isolates with reduced sensitivity to fludioxonil were found in strawberry fields in Virginia (6), Maryland, and South Carolina (8). Low-resistant (LR) and MR *B. cinerea* isolates were reported from European small fruit, including strawberry (20,23) and grapevines (20,22,28). The effective dose that inhibits 50% of mycelial growth (EC₅₀) values for isolates MR to fludioxonil from Germany and Washington were 0.04 to 1.5 µg/ml, depending on the evaluation method (23,43). High levels of resistance to fludioxonil have not been reported in the field, probably due to a fitness penalty in such isolates (4,25).

The precise mode of action of fludioxonil is still unknown but fludioxonil treatment induced cell death in filamentous fungi by improperly activating the Hog1-type mitogen-activated protein kinase (MAPK) (19,41,42), indicating possible disruption of the osmoregulation pathway. High levels of fludioxonil resistance in laboratory mutants are linked to enzymes involved in osmoregulation pathway, such as histidine-kinases (HKs); specifically, the group III HK is considered to be a target for fludioxonil in yeast and filamentous fungi (2,14,30,41). Some evidence was provided for fludioxonil resistance to be linked to at least two different genes (34). Genes assumed to be involved in osmoregulation in *B. cinerea* were identified by gene disruption and include *bos1* (9), *bcsak1* (32), *BcOS4* (40), *bos5* (39), and *BRRG-1* (38). Corresponding knock-out mutants revealed reduced salt tolerance and increased resistance to fludioxonil. Hypersensitivity to osmotic stress has also been associated with lack of glycerol accumulation and resistance to the fludioxonil (18).

Moderate resistance to fludioxonil in *B. cinerea* is associated with the multidrug resistance (MDR) phenotypes MDR1 and MDR1h (20,23). Both phenotypes are associated with overexpression of the ATP-binding cassette (ABC) transporter gene *atrB* and mutations in transcription factor Mrr1 (20,33). Although MDR1 phenotypes express *atrB* ≈50-fold, MDR1h phenotypes express *atrB* at even higher levels (23). The overexpression of two other multidrug transporters has been linked to multifungicide resistance in *B. cinerea*, including *mfsM2* and *atrD*. MfsM2 belongs to the major facilitator superfamily (MFS) and overexpression is associated with increased resistance to multiple fungicides (designated MDR2), including low levels of resistance to fludioxonil (17). AtrD is an ABC transporter that was linked previously to azole resistance in strains of *B. cinerea* (15,16).

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As part of a region-wide fungicide resistance monitoring program involving *B. cinerea* from blackberry and strawberry, we identified several isolates MR and LR to fludioxonil. The goal of this study was to characterize the molecular basis of resistance to fludioxonil in these isolates. Specific objectives were to investigate (i) potential alterations in genes involved in osmoregulation and promoter rearrangements upstream of *mfsM2*, (ii) over-expression of *atrB* and *atrD*, (iii) glycerol synthesis and hypersensitivity to salt stress, and (iv) fitness components and cross resistance with dicarboxamide fungicide iprodione in isolates LR and MR to fludioxonil.

MATERIALS AND METHODS

Isolates and fungicides. *B. cinerea* isolates were obtained from North and South Carolina blackberry fields, a Virginia strawberry field, and Germany (Table 1). Single-spore isolations from symptomatic blackberry and strawberry fruit were conducted as described previously (26). *B. cinerea* strain Do9_K_A31 (designated Do9 in this study) from a strawberry field in Germany was obtained from Dr. Matthias Hahn, University of Kaiserslautern, Kaiserslautern, Germany and used as MDR1h, group S reference strain (23). Formulated fludioxonil (Scholar SC fungicide, 20.4% a.i. [vol/vol]; Syngenta, Research Triangle Park, Raleigh, NC) and iprodione (Rovral 4 FL, 41% a.i. [vol/vol]; Bayer CropScience, Research Triangle Park, NC) were used to determine sensitivities to these fungicides in vitro. Tolnaftate and cycloheximide were purchased from Sigma-Aldrich (St. Louis), dissolved in 100% ethanol, and diluted in ultrapurified water before being added to the liquid medium. Fungicide stock solutions were adjusted to keep final solvent concentrations <1.5% (vol/vol). No significant growth inhibition was observed for strains on media with <1.5% ethanol (20,28).

Fungicide sensitivity tests. Categories of fungicide sensitivity to fludioxonil and iprodione were determined using a spore germination assay (13,36). The EC₅₀ values were determined as described previously (20), with minor modifications. Briefly, 5,000 spores were transferred to 0.1-ml 96-microplate cultures. Final concentrations for all compounds were 10, 3, 1, 0.3, 0.1, 0.03, and 0.01 µg/ml. Tests were performed in malt extract broth. After 48 h of incubation at 22°C in darkness, the optical density at wavelength with absorbance at 600 nm was determined. The experiment was repeated.

DNA extraction, amplification, and sequencing of *mrr1* and the *mfsM2* promoter regions. DNA was extracted as described previously (5). The *mrr1* gene was amplified in two parts; the upstream region was amplified with primers *mrr1_atg* and *TF1-2_new* and the downstream region was amplified with *TF1-3_new* and *TF1-4* (23). The *mfsM2* promoter region of *B. cinerea* was amplified with primers *Prom_Mfs2_1fw* and *Prom_Mfs2_2rev* (20). Fragments were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV-light. The fragment sizes of polymerase chain reaction (PCR) products were verified by comparison with a Low Range Plus DNA Ladder (Fisher Scientific, Waltham, MA). The PCR products were purified using the ExoSAP-IT Purification Kit (USB Corporation, Cleveland, OH) following the manufacturer's recommendations. Purified products were sequenced at Clemson University Genomic Center (Clemson, SC). Nucleotide sequences were analyzed, assembled, and aligned with DNASTAR sequence analysis software (DNASTAR, Inc., Madison, WI). *B. cinerea* strain T4 genome sequences were obtained from GenBank, accession number FQ790286.1.

RNA extraction and gene expression analysis. Isolates were grown on potato dextrose agar (PDA) medium (Difco Laboratories, Sparks, MD) in 9-cm-diameter petri dishes for 10 to 14 days at 22°C with 12-h intervals of fluorescent light and darkness. Conidia were gently scraped off with a sterile, disposable

10-µl inoculation loop (VWR International LLC, Radnor, PA) without touching the agar. Then spores were suspended in 2 ml of potato dextrose broth (PDB), with a final spore concentration of 1 to 5 × 10⁵ in 6-cm-diameter petri dishes. The spores were incubated for 14.5 h on a shaker with 100 rpm at 22°C. The germings were incubated for another 30 min in the petri dish either with or without fludioxonil at a final concentration of 1 mg/liter. For RNA isolation, the germings with the medium were transferred to a 2-ml centrifuge tube and centrifuged for 10,000 rpm for 4 min. The pellet was washed twice with autoclaved, distilled water. Total fungal RNA was isolated using the MasterPure Yeast RNA Purification Kit (EpiCentre Pte. Ltd. Madison, WI) and the RNA quality was checked and quantified by spectrophotometric analysis with a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA). The RNA was then reverse transcribed into cDNA (iScript CDNA Synthesis Kit; Bio-Rad Laboratories, Inc., Hercules, CA). Quantitative reverse-transcription PCR was performed as described previously (13), with the exception that we used primers *atrBfor/atrBrev* and *atrDfor/atrDrev* for *AtrB* and *AtrD* expression analysis, respectively, following published amplification protocols (20). Expression of the genes was calculated according to Pfaffel (31). Transcript levels were normalized against the expression levels of housekeeping genes encoding elongation factor 1α (BC1G_09492.1) and shown as normalized fold-expression levels of noninduced germings from sensitive (S) strains. Means of at least two biological replicates are shown. Three technical replicates of PCR were used for each biological replicate.

Salt tolerance and determination of glycerol content in germings. Salt tolerance was investigated on 4% NaCl-amended minimal medium. A 5-mm-diameter mycelial plug taken from a 3-day-old culture grown on PDA was transferred to the center of the amended medium and dishes were incubated at 22°C in the dark. The colony diameter was measured after 3 days of incubation with three replicates per isolate. Mean colony diameter was used to calculate mycelia growth inhibition rate. The experiment was repeated.

The glycerol content was determined in germings of S, LR, and MR isolates to assess involvement of osmoregulation in the resistance mechanism. Conidia were suspended in 2 ml of PDB in 6-cm-diameter petri dishes with a final concentration of 1 × 10⁵ conidia/ml. Four dishes per isolate per treatment were incubated for 48 h on a shaker at 100 rpm and 22°C. The germings were incubated for another 1.5 h in the dishes either with or without fludioxonil at a final concentration of 1 mg/liter. The medium containing the germings was transferred to a 2-ml impact-resistant screw-cap tube (USA Scientific, Inc. Orlando, FL) and centrifuged at 12,000 rpm for 10 min. Medium was removed and the pellet was washed twice with autoclaved distilled water. The volume for each sample was adjusted to 1 ml with water and ground at 4 m/s for 40 s using a FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals, Solon, OH). Samples were then centrifuged for 10 min at 12,000 rpm and the glycerol content in the supernatant was determined using an EnzyChrom Glycerol

TABLE 1. Name, origin, and host of isolates used in this study

Isolate name	Origin	Host	Year of isolation
CA25	South Carolina	Blackberry	2010
CB3a	South Carolina	Blackberry	2011
CO5	South Carolina	Blackberry	2010
MC14	South Carolina	Blackberry	2010
WM14	South Carolina	Blackberry	2010
CO3a	South Carolina	Blackberry	2011
CB82a	South Carolina	Blackberry	2011
Wland1	Virginia	Strawberry	2012
KC25	North Carolina	Blackberry	2011
KC33	North Carolina	Blackberry	2011
Do9_K_A31	Germany	Strawberry	2009

Assay Kit, following the manufacturer's instructions. After all of the supernatant was removed, the precipitated cell debris was completely dried in an oven for 24 h at 60°C. The experiments were repeated two times.

Detection of mutations in the *bcsak1*, *BcOS4*, *bos5*, and *BRRG-1* genes. Nucleotide sequences of osmotic regulator genes *bcsak1* (32), *BcOS4* (40), *bos5* (39), and *BRRG-1* (38) were amplified by PCR from fludioxonil S, LR, and MR isolates. Two overlapping fragments of *bcsak1* were amplified with primer pairs SAK1F-f (TACCCACTCAACCACCAAC)/SAK1F-r (ATCTTG AATACGGGCGAG) and SAK1B-f (GCTGGTCCTGGAGATA CTAAGA)/SAK1B-r (AAGCACAAGAGAGCACTCCT). These two fragments were assembled into one contiguous sequence covering the entire gene. The core sequence of *BcOS4* was amplified with primer pair BOS4C-f (GCTGACGATGAGG AATCA)/BOS4C-r (GTGCTGTAAACACCGACA). The primers were designed based on the *B. cinerea* T4 genome sequences (1). The *bos5* and *BRRG-1* genes were amplified with primer pairs Os5-F1/Os5-R1 (39) and Rrg-F1/Rrg-R1, respectively (38).

Assessment of fitness and pathogenicity on fungicide-treated fruit. The following fitness components were investigated for isolates sensitive and resistant to fludioxonil and iprodione. All experiments were repeated.

Mycelial growth on PDA. PDA dishes were inoculated with a 5-mm-diameter mycelial plug taken from 7-day-old culture grown on PDA. The dishes were incubated at 22°C in the dark. The colony diameter was measured after 3 days of incubation with three replicates per isolate. Mean colony diameter was calculated for growth rate determination.

Pathogenicity on untreated and fungicide-treated detached fruit. Pathogenicity of each isolate was confirmed by determining its ability to infect and sporulate on strawberry fruit. Commercially grown ripe strawberry fruit were rinsed with sterile water three times for 30 s each and allowed to air dry. Then, they were placed into plastic boxes (eight strawberry fruit per box for each of the three replicates). When the fruit surface had dried, each fruit was punctured at one point to a depth of 9.5 mm using a 26G3/8 9.5-mm beveled syringe tip (Becton Dickinson & Co.). The wounds were injected with a 30- μ l droplet of conidia suspension (10^6 spores/ml) prepared in distilled sterile water using the same type of syringe. Most of the conidia suspension formed a droplet on top of the wounded area. After inoculation, the boxes were kept at 22°C. During the first 24 h, the boxes were sealed with plastic bags to keep the relative humidity high. After 4 days, the lesion diameters were measured.

The ability of isolates resistant to fludioxonil and iprodione to cause disease on fruit treated with fungicides was assessed. Experiments were performed as described above for pathogenicity, tests with minor modifications. Strawberry fruit were sprayed 4 h prior to inoculation with the recommended label rate of the fungicides Scholar SC (2.5 ml/liter), Switch 62.5WG (1.9 g/liter; combination of fludioxonil 25% [vol/vol] and cyprodinil 37.5% [vol/vol]), and Rovral 4 Flowable (2.5 ml/liter), using a hand mister to run off. Control fruit were sprayed with sterile distilled water. After 4 days of inoculation, the absence or presence of sporulating lesions was assessed and the lesion diameters were measured and calculated as percentage of the control. The experiment was repeated.

Data analysis. Statistical analysis was performed related to the in vitro and in vivo responses (growth rate, lesion size, disease incidence, control efficacy, and relative expression of *atrB* and *atrD* genes) to the experimental factors of experimental replication, isolate, or fungicide treatment. Significant differences were determined through Tukey's honestly significant difference test. All calculations were performed by IBM SPSS Statistics (version 19; IBM SPSS, Armonk, NY) and all tests were performed with $\alpha = 0.05$, except as described otherwise.

RESULTS

Sensitivity of isolates to fludioxonil and iprodione in vitro.

In our collection of 412 *B. cinerea* isolates from blackberry and strawberry, only four isolates were resistant to fludioxonil. Two (Wland1 and CB82a) were categorized as LR based on their ability to germinate with fludioxonil at 1 μ g/ml and two (KC25 and KC33) as MR based on their ability to germinate with fludioxonil at 1 μ g/ml and show residual growth at 10 μ g/ml. EC₅₀ values determined in microtiter assays were 0.16 and 0.26 μ g/ml for LR isolates and 0.32 and 0.38 μ g/ml for MR isolates. Isolates LR or MR but not S to fludioxonil were R to tolnaftate and all were S to cycloheximide, consistent with the MDR1 phenotype. Together with two S isolates, the four LR and MR isolates were also investigated for iprodione sensitivity because phenylpyrroles and dicarboxamides are both believed to target proteins involved in osmotic regulation. All isolates LR and MR to fludioxonil were also R to iprodione but some fludioxonil-sensitive isolates were LR to iprodione (Table 2).

Expression of multidrug transporter genes *atrB* and *atrD* in isolates with different fludioxonil sensitivity phenotypes. The *atrB* genes of MR isolates from blackberry (KC25 and KC33) and

TABLE 2. Fitness components for *Botrytis cinerea* isolates sensitive (S), low-resistant (LR), moderately resistant (MR), or resistant to fludioxonil or iprodione

					Fitness components ^z								
					4 dai								
Phenotype ^x			EC ₅₀ (mg/liter) ^y		3 dai	LS (cm)					SL (%)		
Isolates	Fludioxonil	Iprodione	Fludioxonil	Iprodione	MG (cm)	LS (cm)	SL (%)	Scholar	Switch	Rovral	Scholar	Switch	Rovral
CA25	S	S	n/d	n/d	7.3 b	3.3 b	100.0	0.0 a	n/d	0.01 a	0.0	n/d	4.2 a
CB3a	S	S	0.09	0.21	7.3 ab	3.3 b	100.0	0.0 a	0.0	0.01 a	0.0	0.0	2.7 a
CO5	S	LR	n/d	n/d	7.6 b	3.0 b	100.0	0.0 a	n/d	2.0 bc	0.0	n/d	89.6 b
MC14	S	LR	0.06	3.09	7.4 b	3.5 b	100.0	0.0 a	n/d	2.5 bcd	0.0	n/d	100.0 b
WM14	S	LR	n/d	n/d	7.4 b	3.3 b	100.0	0.0 a	n/d	1.9 b	0.0	n/d	100.0 b
CO3a	S	HR	n/d	n/d	7.2 ab	3.5 b	100.0	0.0 a	0.0	2.7 de	0.0	0.0	100.0 b
CB82a	LR	HR	0.16	6.94	7.5 b	3.2 b	100.0	1.2 b	0.0	2.5 cd	100.0	0.0	100.0 b
Wland1	LR	HR	0.26	3.56	7.1 ab	2.7 a	100.0	1.7 c	n/d	2.2 bcd	100.0	n/d	100.0 b
KC25	MR	HR	0.32	>10	6.1 a	2.8 a	100.0	1.1 b	1.8	2.4 bcd	100.0	98.3	97.9 b
KC33	MR	HR	0.38	>10	7.3 b	3.3 b	100.0	1.8 c	1.5	3.2 e	100.0	100.0	100.0 b

^x Phenotypes were determined based on a spore germination assay using fludioxonil at 0.1 and 10 μ g/ml and iprodione at 5 and 50 μ g/ml (36). The phenotypic characterization (S, LR, MR, or HR) for some of the isolates listed was reported in an earlier study from our lab (13).

^y Fungicide concentration that reduced mycelial growth by 50%.

^z Abbreviations: LS = lesion size (diameter), SL = sporulating lesions, MG = mycelial growth, dai = days after inoculation or transfer, and n/d = not determined. Numbers in each column followed by the same letter are not significantly different at $\alpha = 0.05$ as determined by analysis of variance. Mean separation was conducted using Tukey's test.

MDR1h reference isolate Do9 were expressed at ≈ 200 -fold the level of the S isolates (Fig. 1). Lower expression levels were found for all other phenotypes. The *atrB* expression levels of LR isolates Wland1 and CB82a were 30- and 100-fold increased, respectively, compared with the S isolate (Fig. 1). Fludioxonil exposure prior to RNA extraction increased expression of four of the six isolates regardless of their phenotype. They included S isolate CB3a, LR isolate Wland1, and MR isolates KC33 and MDR1h reference isolate Do9.

Analysis of *atrD* gene expression was conducted because previous reports noted overexpression of this gene in association with MDR phenotypes (15,16). In this study, the expression of *atrD* was not different among isolates S, LR, and MR to fludioxonil in untreated or fludioxonil-treated germlings (Fig. 2). Interestingly, the expression of *atrD* in treated germlings was reduced by $>50\%$ in all isolates after fludioxonil treatment.

Mutations in the transcription factor *mrr1* and the promoter sequences of *mfsM2* in strains LR or MR to fludioxonil. Certain mutations in transcription factor Mrr1 influence *atrB* expression. Therefore, *mrr1* was sequenced in its entirety from

strawberry isolate Wland1 and six blackberry isolates representing S and R phenotypes. The *mrr1* coding regions (including introns) of two S isolates from blackberry (MC14 and KC20) were 2,444 bp in length and encoded identical amino acid sequences compared with reference isolate T4, with the exception of two amino-acid variations (L475S and M483V) in isolate MC14 (Fig. 3). S isolate CB3a had an *mrr1* gene sequence of 2,477 bp in length and revealed several amino-acid variations compared with T4. The *mrr1* coding region of isolates with resistance phenotypes were 2,444, 2,483, and 2,489 bp in size for isolates Wland1, CB82a, and KC25 and KC33 (both 2,489 bp), respectively. Two insertions characteristic for group S isolates, 18 and 21 bp in length, were discovered in one S, the LR, and both MR isolates from blackberry (Fig. 3). Group S isolates were considered a special clade of *B. cinerea* and genetically different from the isolates studied (23). In our study, there were only five amino-acid variations among non-group S isolates but considerably more (≈ 13 , on average) among group S isolates. The most diversity (up to 38 amino-acid variations) among *mrr1* gene sequences was found between non-group S and group S isolates.

Fig. 1. Expression analysis of *atrB* determined by quantitative reverse-transcription polymerase chain reaction. Values indicate expression levels relative to the expression of sensitive strain CB3a (which was normalized to 1) with (shaded bars) and without (white bars) fludioxonil treatment prior to RNA extraction.

Fig. 2. Expression analysis of *atrD* determined by quantitative reverse-transcription polymerase chain reaction. Values indicate expression levels relative to the expression of sensitive strain CB3a with (shaded bars) and without (white bars) fludioxonil treatment prior to RNA extraction.

The MDR1-associated mutation R632I was the only mutation uniquely associated with the fludioxonil MR phenotype (Fig. 3). No point mutations or insertions were detected in the *mfsM2* promoter region of isolates LR or MR to fludioxonil, indicating that the isolates did not belong to the MDR2 or MDR3 genotype. The *mrr1* sequences of isolates CB3a, MC14, KC20, CB82a, KC25, and KC33 were submitted to GenBank (accession numbers KJ569562, KJ569563, KJ569564, KJ569565, KJ569566, and KJ569567, respectively).

Evaluation of salt tolerance and glycerol content. Changes in osmoregulatory pathways conferring resistance to phenylpyrroles and dicarboxamides may also influence osmotic sensitivity (11, 17,29,37). Isolates MR to fludioxonil were significantly more susceptible to salt stress compared with isolates LR and S to fludioxonil (Fig. 4). The sensitivity varied among S isolates, with a range of 25 to 40% inhibition. One of each fludioxonil sensitivity phenotype (S, LR, and MR) was subjected to a glycerol content analysis. The three representative isolates roughly produced similar amounts of glycerol in germlings (Fig. 5). Exposure of germlings to fludioxonil prior to extraction increased glycerol content approximately fourfold in all isolates regardless of the phenotype.

Sequencing of genes involved in the osmotic signal transduction pathway. We sequenced large portions of the *bcsak1* (1,669 bp), *bos5* (1,880 bp), and *BRRG-1* (1,655 bp) genes and the core region of the *BcOS4* (1,091 bp) gene for the presence of point mutations in S, LR, and MR isolates. No point mutations were identified that were uniquely associated with the fludioxonil

LR or MR phenotype (data not shown). Both MR isolates had a 5-bp deletion in an intron at nucleotide position 1,261 of the *bcsak1* gene (data not shown); the sequences from one S and one MR isolate were submitted to GenBank (accession numbers KF964016 [CB3a] and KF964017 [KC25]). Analysis of 45 fludioxonil-sensitive isolates that originated from the same site as the MR isolates (location KC), and 11, 16, 4, and 24 isolates from locations MC, CO, CA, and WM, respectively, revealed that the deletion was unique to the MR isolates (data not shown). However, the deletion was absent in the reference strain from Germany MR to fludioxonil, suggesting that the deletion is not linked to the MDR1 phenotype.

Fitness components of isolates S, LR, and MR to fludioxonil. Cross resistance between phenylpyrroles and dicarboxamides is common because products of both fungicide classes are believed to be involved in the inhibition of proteins involved in osmotic signal transduction. All isolates LR and MR to fludioxonil were also R to iprodione. The fludioxonil-sensitive isolates however, revealed various iprodione sensitivity phenotypes (Table 2). There were no significant differences in mycelial growth rates and lesion size between S isolates and LR and MR isolates, with one exception. Mycelium growth was slower for isolate KC25 compared with most isolates, including four S and two dual-R isolates (Table 2). The same isolate also produced a significantly smaller lesion ($P < 0.05$) size on inoculated fruit compared with all other isolates. The other MR isolate, KC33, did not suffer a fitness penalty in our study. All S and R isolates were able to produce sporulating lesions on detached fruit (Table 2).

Fig. 3. Amino acid variations found in the transcription factor Mrr1 of isolates from strawberry and blackberry with different fludioxonil resistance phenotypes. Nucleotide insertions are indicated by '+' followed by the number of inserted base pairs. Nucleotide deletions are indicated with 'Δ' followed by the number of missing base pairs. *Botrytis cinerea* group S isolates are marked with '*' after the isolate name. Vertical lines indicate amino-acid variations between group S and non-group S isolates. If the change was consistent among all isolates from the group S, the exact amino-acid change is not indicated for simplicity reasons. The number of horizontal lines attached to the bottom of a vertical line corresponds to the number of amino-acid variations at that position that are different from non-group S isolates. The mutation R632I associated with MDR1 is indicated in bold.

The ability of isolates S, LR, and MR to fludioxonil to cause disease on fruit sprayed with fungicides was assessed to determine whether these isolates can withstand label rates of fungicide. Isolates S to fludioxonil were completely controlled with Scholar SC fungicide, but isolates LR and MR to fludioxonil developed disease with sporulating lesions. Both isolates MR to fludioxonil were also able to develop sporulating lesions on fruit treated with Switch 62.5WG, a commercially available combination product of fludioxonil and AP fungicide cyprodinil, indicating that the AP fungicide component in this mixture did not prevent disease formation. This is consistent with the in vitro resistance to cyprodinil and fludioxonil and the MDR1 phenotype. Isolates R to iprodione in vitro, including the isolates R to both iprodione and fludioxonil, developed disease with sporulating lesions on fruit treated with label rates of Rovral 4 Flowable. The isolates S to iprodione were almost completely controlled in the Rovral 4 Flowable treatment (Table 2).

DISCUSSION

Although resistance in *B. cinerea* to many classes of fungicides, including benzimidazoles, quinone outside inhibitors, succinate

dehydrogenase inhibitors, hydroxanilides, anilinopyrimidines, and dicarboxamides, is fairly common (3,21–23,27,35), resistance to phenylpyrroles, including fludioxonil, is still rare. In this study, we show that resistance to fludioxonil is emerging in small fruit crops in the eastern United States. Resistance to fludioxonil is likely to increase in light of resistance problems with many other compounds of different modes of action. The low frequencies of isolates LR and MR to fludioxonil in our collection of 412 isolates from blackberry and strawberry is consistent with other studies that found that fludioxonil resistance is either absent or rare in southeastern strawberry fields (6,7) and that it is likely not of practical relevance for fruit growers at this time. The low frequency of LR and MR isolates validates the idea that phenylpyrroles are still valuable tools for rotations or mixtures with other site-specific fungicides for effective gray mold control. However, the here-documented emergence of such strains in United States berry crop production fields should be considered in disease management strategies meant to delay selection. Examples already exist where large portions of field isolates gained partial resistance to fludioxonil due to fungicide selection (23).

In this study, the EC₅₀ values for fludioxonil in MR isolates were comparable with those found in isolates from strawberry

Fig. 4. Mycelial growth inhibition of isolates sensitive, low-resistant, and moderately resistant to fludioxonil on minimal medium amended with 4% NaCl.

Fig. 5. Glycerol content in mycelia of *Botrytis cinerea* isolates sensitive, low-resistant, and moderately resistant to fludioxonil. Mycelia subjected to fludioxonil treatment prior to analysis are indicated in shaded bars.

(20,23) and grapevines (20,22,28) in Europe. Moderate resistance to fludioxonil in *B. cinerea* isolates from European grape was designated MDR1 and conferred by mutation R632I in transcription factor Mrr1, leading to overexpression of ABC transporter gene *atrB* (20). Both MR isolates analyzed in this study contained the R632I mutation in Mrr1 associated with MDR1 but *atrB* overexpression levels were similar to MDR1h reference isolate Do9 (20,33). MDR1h isolates revealed higher levels of resistance to cyprodinil and fludioxonil with generally higher overexpression of *atrB* compared with MDR1 (23). Our isolates, however, did not possess the MDR1h's signature L497 deletion in Mrr1 (23). This suggests that this deletion may not exclusively be responsible for the high levels of *atrB* overexpression. MR isolates were R to tolnaftate but S to cycloheximide, further confirming the MDR1 phenotype (20). The existence of MDR1 in the United States suggests that this resistance mechanism is important for the adaptation of *B. cinerea* to fludioxonil pressure. Because of the great distance between the two continents, the Atlantic Ocean separating the two land masses, and the significant genetic variation in the *mrr1* gene between the German MDR1h reference strain and the isolates from the United States (data not shown), it is likely that the same resistance mechanism developed independently in the two continents.

The LR phenotype may be caused by overexpression of *atrB* as well, albeit at a lower expression level (up to 100-fold in this study). However, variations associated with the MDR1 or MDR1h phenotypes were not found in the *mrr1* genes of LR isolates. An investigation into genes involved in osmotic regulation, including *bcsak1*, *bos5*, *BcOS4*, and *BRRG-1*, revealed no nucleotide variations associated with resistance between S, LR, or MR isolates. Furthermore, we found no evidence in MR or LR isolates that would suggest involvement of the MDR2 genotype, which is characterized by promoter region rearrangements upstream of the *mfsM2* promoter region and reduced sensitivity to cycloheximide (data not shown) (20).

The lack of fitness cost of isolate KC33, MR to fludioxonil, in this study indicates that overexpression of *atrB* may not necessarily come with a fitness cost. However, MR isolate KC25 did have slower growth rates on fruit, which is consistent with reports for a fludioxonil-resistant *Penicillium expansum* field isolate (25). Laboratory mutants of *B. cinerea* revealed two types of fludioxonil-resistant phenotype; one R to osmotic stress (FLD_{osm/r}) and another S to high osmotic stress (FLD_{osm/s}). Only FLD_{osm/s} had fitness cost (44) that was later confirmed in field studies (43). In this study, both MR isolates were hypersensitive to salt in the form of exposure to 4% NaCl despite having different fitness phenotypes. Neither salt-stress hypersensitivity nor a linkage to iprodione resistance has been reported for MDR1 and MDR1h strains before, and are not necessarily expected because MDR phenotypes are purely based on efflux transporter overexpression. Whether hypersensitivity to salt stress and MR to fludioxonil are linked is unknown due to the low sample size investigated in this study. If they are linked, it could be exploited for management purposes. The increased sensitivity to salt stress was not associated with mutations in the *bcsak1*, *bos5*, *BRRG-1*, and *BcOS4* genes involved in the osmoregulation pathway (data not shown). It is possible that their expression pattern or other genes involved in osmoregulation are responsible for this phenotype. Increased sensitivity to salt stress was also found in *Botrytis* isolates from apple orchards in Washington State (43). Numerous studies reported the linkage of the fludioxonil-resistant phenotype and hypersensitivity to salt stress in laboratory mutants with dysfunctional genes involved in osmoregulation in *Neurospora crassa* (10,11,29) as well as in *B. cinerea* (9,32,38–40). Previous studies on the mode of actions of phenylpyrrole fungicides in *N. crassa* indicated that this class of fungicides affects the osmotic signal transduction pathway (10) and our results indicate that this mode of action is also true for *B. cinerea*.

All isolates, regardless of fludioxonil resistance phenotype, accumulated glycerol when exposed to fludioxonil, which increases the internal turgor pressure. In other fungi, the exposure to fludioxonil increased the intracellular glycerol content for both S and MR isolates but not highly R isolates in *P. digitatum* (18). The authors concluded that the mode of action of fludioxonil in *P. digitatum* is probably the MAPK pathway that stimulates glycerol synthesis in S and MR. We saw the same phenomenon in our S, LR, and MR isolates, which confirms the involvement of fludioxonil in the MAPK pathway in *B. cinerea* but also suggests that the mechanism conferring LR and MR may not be directly associated with the regulation of glycerol synthesis.

We focused our *mrr1* gene analysis on characterization of fludioxonil phenotypes and therefore sequenced three S, two LR (one from blackberry and one from strawberry), and two MR isolates. Among those strains, one S, one LR (blackberry), and two MR contained the *B. cinerea* group S signature consistent with two insertions (18 and 21 bp) in *mrr1* (Fig. 3), which were first identified in isolates from German strawberry fields (23). Thus far, group S isolates from Germany have been exclusively found in strawberry fields and are believed to be a result of differential host adaptation or reduced genetic exchange (23). The presence of group S and non-group S strains in populations from blackberry in this study would indicate that group S strains are not a result of host adaptation. Group S strains might be sexually isolated from other clades as suggested above, given the large numbers of amino-acid variation in group S compared with the non-group S strains. However, many more isolates and different genes would need to be sequenced to validate this hypothesis.

The LR and MR phenotypes were capable of developing disease on fruit sprayed with label rates of Switch 62.5WG, Scholar SC, or Rovral 4 Flowable. Together with our fitness data, this indicates that these resistance phenotypes are likely being selected for by these fungicides in the field and that they may be able to compete with S isolates in the absence of selection pressure. This finding is consistent with a study from Germany that showed establishment of MDR-like phenotypes in both vineyards and strawberry fields after application of different spray programs containing Signum (pyraclostrobin and boscalid), Teldor (fenhexamid), and Switch 62.5WG (23).

In conclusion, *B. cinerea* strains with resistance to fludioxonil are emerging in strawberry and blackberry fields of the southern United States. The mechanism of resistance in MR isolates is the same as described in European isolates, emphasizing the importance and relevance of this mechanism of resistance for fludioxonil. However, the low number of isolates examined does not allow firm conclusions regarding to what extent this mechanism of resistance is present in MR isolates. The role of *atrB* overexpression in LR isolates is still unclear.

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