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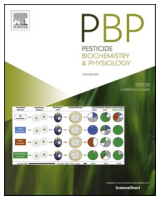

Inherent tolerance of *Colletotrichum gloeosporioides* to fludioxonil

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A B S T R A C T

Colletotrichum spp. cause devastating diseases in agricultural crops, including fruit crops. They can differ in host plant and plant organ specificity and even in fungicide sensitivity. In strawberry, members of the *C. gloeosporioides* species complex (referred to as *C. gloeosporioides*) primarily cause crown rot and those of the *C. acutatum* species complex (referred to as *C. acutatum*) primarily cause fruit rot. Fludioxonil is registered for use (in combination with cyprodinil; Switch 62.5WG in the US) in strawberry against anthracnose disease caused by *Colletotrichum* spp. In this study we examined the sensitivity of *C. gloeosporioides* (*C. fructicola* and *C. siamense*) and *C. acutatum* (*C. nymphaeae* and *C. fioriniae*) isolates from different hosts and different geographical locations in the US to fludioxonil and examined possible mechanisms of inherent fungicide tolerance. The dose response to fludioxonil of *C. gloeosporioides* isolates (including 4 isolates of *C. theobromicola*) revealed about 70% inhibition of mycelial growth at 1 mg/L that was maintained at 10 mg/L and 100 mg/L and lead to minimum inhibitory concentration (MIC) values >100 mg/L. In contrast, mycelial growth of *C. acutatum* isolates was completely inhibited at 1 mg/L. *C. gloeosporioides* isolates were also significantly less sensitive to iprodione. An investigation into possible mechanisms of *C. gloeosporioides* isolates tolerance to fludioxonil and iprodione revealed no evidence of *OS-1* gene involvement. Isolates of both species complexes were equally sensitive to salt stress based on mycelial growth inhibition on potato dextrose agar amended with 2%, 4%, and 6% NaCl. In addition, orthologous amino acid alterations in OS-1 previously linked to fludioxonil resistance in *Botrytis cinerea* were not found in *C. gloeosporioides* or *C. acutatum* isolates. This study also showed limited *in vitro* inhibitory activity of cyprodinil against isolates of both species complexes (MIC values >100 mg/L) and unveils a potential weakness of the fludioxonil+cyprodinil premixture marketed as Switch 62.5WG against *C. gloeosporioides* species complexes.

1. Introduction

Colletotrichum spp. are fungal plant pathogens that can cause grave economic losses to field crop, fruit, and vegetable producers. In temperate climates *C. acutatum* and *C. gloeosporioides* species compexes cause most of the damage in pome fruits, stone fruits, and small fruits production (Dowling et al., 2020). In strawberries, *C. acutatum* species complexes are generally responsible for fruit rots. The most important one being *C. nymphaeae,* the main species affecting the fruit and causing fruit anthracnose but it can be found on roots and stems as well (Wang et al., 2019). Another species belonging to the same species complex and affecting strawberry fruit is *C. fioriniae* in the US (Dowling et al., 2020). *C. fructicola* and *C. siamense* belong to the *C. gloeosporioides* complex and affect primarily the crown and cause crown rot but can be found on rare occasions on roots, stems, and fruit (Peres et al., 2005). Fruit anthracnose caused by *C. acutatum* and crown rot caused by *C. gloeosporioides* affect southeastern strawberry production in the US. Breeding programs are making progress integrating resistance genes into new cultivars (Salinas et al., 2019), but very few anthracnose-tolerant cultivars have been adopted to mainstream production.

Despite the economic threat of diseases caused by *Colletotrichum* spp., very few fungicides have been developed with high efficacy against *Colletotrichum* species. In strawberries, management of anthracnose fruit rot is primarily based on captan, quinone outside inhibitors (QoIs; *e.g.* azoxystrobin, pyraclostrobin, and trifloxystrobin), and phenylpyrroles (PP; fludioxonil) (Brannen et al., 2019). Captan is a multisite fungicide applied at relatively high dose and is considered the weakest of the three chemical classes. The QoIs are highly effective but after more than 20 years of exposure resistance in *C. nymphaeae* has emerged and is now widespread in US east coast production areas (Forcelini et al., 2016; Forcelini and Peres, 2018). As a result, many growers rely on fludioxonil for *Colletotrichum* species-associated disease management.

Fludioxonil is sold in combination with cyprodinil in form of Switch 62.5WG. The combination serves to broaden the spectrum of efficacy but also to slow down the emergence and selection of resistance to either active ingredient. The current Switch 62.5WG label recommends the product to be used for anthracnose disease control of strawberry caused by *Colletotrichum* spp. Preliminary data obtained in our laboratory indicated significant differences in sensitivity to fludioxonil between *C. acutatum* isolates and *C. gloeosporioides* isolates suggesting that Switch 62.5WG may not be effective against all *Colletotrichum* spp. affecting strawberry. Therefore, the objective of this study was to determine the

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sensitivity of *C. acutatum* and *C. gloeosporioides* to fludioxonil and to cyprodinil and to investigate possible mechanisms of intrinsic tolerance.

2. Materials and methods

2.1. Fungal isolates and culture conditions

Sixteen isolates of *Colletotrichum* spp. including 3 isolates of *C. fioriniae* and 3 isolates of *C. nymphaeae* of the *C. acutatum* species complex and 6 isolates of *C. siamense* and 4 isolates of *C. fructicola* of the *C. gloeosporioides* species complex were recovered from dried mycelium on filter paper stored at 4 °C at Clemson University. Their designation, origin, host, year of isolation, and past references are listed in Table 1. An additional set of 4 *C. siamense*, 4 *C. fructicola*, and 4 *C. theobromicola* isolates (All belong to the *C. gloeosporioides* species complex) was recovered from storage and only used for fludioxonil sensitivity determination (not shown in Table 1). We will refer to *C. acutatum* species complex as *C. acutatum* and to the *C. gloeosporioides* species complex as *C. gloeosporioides*.

2.2. Characterization of sensitivity to fludioxonil and iprodione

Four mm plugs were transferred from the margins of 5-day old cultures grown on potato dextrose agar (PDA) to fungicide-amended media and colony diameters were measured after 3 days of incubation at 22 °C in the dark. Mycelial growth tests were performed on PDA either unamended or amended with 1, 10, and 100 mg/L fludioxonil (Scholar SC Fungicide 20.4 %; Syngenta, Greensboro, NC, USA) or iprodione (Rovral 4 Flowable Fungicide 41.6 %; BayerCrop Science, Research Triangle Park, NC, USA). Sensitivity to cyprodinil (Vangard WG 75.0 %; Syngenta) was investigated at the same doses on Czapek-Dox medium (CZA) medium (Malandrakis et al., 2015). Sensitivity to cycloheximide (obtained from Fisher Scientific Products, Pittsburgh, PA) was assessed on PDA at 0, 0.01, 0.1, 1.0, 10, and 100 mg/L. Two replicate plates were used for each fungicide concentration and media type and the experiments were conducted twice.

2.3. DNA extraction, amplification, and sequencing of OS-1 genes

Fungal DNA was extracted using a quick and safe method (Chi et al., 2009). Because the *OS-1* gene has been associated with high fludioxonil resistance in plant pathogens (Ren et al., 2016; Sang et al., 2018; Zhou et al., 2020), the gene's sequence of *C. fioriniae* PJ7 of the *C. acutatum* complex (UniProt; T0JSQ2) and *C. gloeosporioides* Cg-14 (UniProt; A0A010RRG8) were downloaded and compared with *Botrytis cinerea* B05.10 Bos1 (GenBank; XP_024546375). In addition, *OS-1* gene fragments from six *C. acutatum* isolates (Ebel1, SC_A6, Ebel2, OH_S1, OH_S2, and AAU811-3) and five *C. gloeosporioides* isolates (SC_A2, IL_A02, IL_A03, KNT520-3, and 19002) were sequenced and orthologous amino acid positions examined. *OS-1* gene fragments from *C. acutatum* isolates were amplified using CaOs1-1F & CaOs1-1R (5′-TCTGGAGCGGGAACTATGGA-3′; 5′-GTCGAGATACTGCGGACCTG-3′) and CaOs1-2F & CaOs1-2R (5′-GGTCCGCAGTATCTCGACTG-3′; 5′-GTGACCCTTGTACGGCTTGA-3′). Gene fragments from *C. gloeosporioides* were amplified using CgOs1-1F & CgOs1-1R (5′-TTTCTGCAACGAAGTGCAGC-3′; 5′-CGGAACGAATCGCCAATGAC-3′), CgOs1-3F & CgOs1-3R (5′-CCAGACGACAGTCAACGTCA-3′; 5′-GCCATTCGCAATAGCCTGTG-3′), CgOs1-5F & CgOs1-5R (5′-CTCAGGTCGACAACGTGGAA-3′; 5′-TGTGTCAAGTCCGTGTCGAG-3′), CgOs1-6F & CgOs1-6R (5′-CATTGGCGATTCGTTCCGTC-3′; 5′-TCTTGACTGCAAGCCTCTGG-3′), and CgOs1-7F & CgOs1-7R (5′-GAGGTCGGAACCGAAGGTTT-3′; 5′-TGACGTTGACTGTCGTCTGG-3′). The fragments amplified covered all but two codons (exceptions were A1259 and T1267) previously linked to fludioxonil resistance (Ren et al., 2016; Sang et al., 2018; Zhou et al., 2020). Amplification was performed using the following parameters: initial denaturation at 95 °C for 3 min; followed by 34 cycles at 94 °C for 5 min, 60 °C for 1 min, and 72 °C for 1.5 min, with final extention at 72 °C for 5 min. The annealing temperature was adjusted depending on the primer pair used: 60 °C for CaOs1-1F & CaOs1-1R and CaOs1-2F & CaOs1-2R, 59 °C for CgOs1-1F & CgOs1-1R, and 57 °C for CgOs1-5F & CgOs1-5R, CgOs1-6F & CgOs1-6R, and CgOs1-7F & CgO1-7R, respectively. PCR products were purified and sequenced by Arizona State University CORE laboratories. Resulting DNA sequences were aligned and translated, and then translations were aligned using SEQman Pro, SEQbuilder Pro (DNASTAR, Inc.; Madison, WI), and MEGA7, respectively.

2.4. Mycelium osmotic sensitivity determination

To determine if low sensitivity to fludioxonil in *C. gloeosporioides* isolates is linked to osmoregulatory pathways, the sensitivity to osmotic stress was determined. Isolates were grown on PDA for 5 days before 4 mm plugs were transferred from colony margins to PDA amended with 0%, 2%, 4%, and 6% NaCl, with 2 replicate plugs for each concentration. After 3 days, relative growth was determined. The experiment was repeated.

2.5. Data analysis

All *in vitro* experiments were conducted two times and the 95% confidence interval (CI) of mean values, calculated by Microsoft Office Excel (Seattle, WA), was compared. Statistical differences were found

Table 1
List of *Colletotrichum* spp. isolates used in this study.

Species Complex	Species	Isolate	Origin	Host	Year of isolation	Reference
C. gloeosporioides	*C. fructicola*	SC_A2	South Carolina, USA	Apple	2018	Chechi et al., 2019
		19002	Nara, Japan	Strawberry	2007	Ishii et al. 2016
		19009	Nara, Japan	Strawberry	2007	MAFF* 306783
		KNT520-3	Tochigi, Japan	Strawberry	1999	This study
	C. siamense	IL_A01	Illinois, USA	Apple	2018	Chechi et al., 2019
		IL_A02	Illinois, USA	Apple	2018	Chechi et al., 2019
		IL_A03	Illinois, USA	Apple	2018	Chechi et al., 2019
		FL98-139	Florida	Strawberry	1998	Hu et al. 2015
		FL99-21	Florida	Strawberry	1999	Hu et al. 2015
		19024	Nara, Japan	Strawberry	2007	MAFF* 306785
C. acutatum	*C. nymphaeae*	Ebel 2	South Carolina, USA	Tomato	2018	Chechi et al., 2020; Chechi et al., 2019b
		OH_S1	Ohio, USA	Strawberry	2018	Chechi et al., 2020
		OH_S2	Ohio, USA	Strawberry	2018	Chechi et al., 2020
	C. fioriniae	Ebel 1	South Carolina, USA	Tomato	2018	Chechi et al., 2019b
		SC_A6	South Carolina, USA	Apple	2018	Chechi et al., 2019
		AAU811-3	Tochigi, Japan	Strawberry	1999	Ishii et al. 2016

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when the 95% CI of the means did not overlap.

3. Results

3.1. Sensitivity of Colletotrichum spp. to fludioxonil and iprodione

The response of *C. gloeosporioides* isolates to fludioxonil and cyprodinil was not linear (Fig. 1) and consequently the minimum inhibitory concentration (MIC) values rather than the 50% effective concentration (EC_{50}) values are reported. *C. gloeosporioides* isolates continued to grow even at the highest fludioxonil concentration at a rate of about 30%. An additional set of 12 *C. gloeosporioides* isolates, including 4 *C. theobromicola* isolates supplied by the University of Florida, revealed the same sensitivity pattern (data not shown). *C. acutatum* isolates were highly sensitive to fludioxonil and were completely inhibited at 1 mg/L. However, isolates of both species complexes continued to grow at a rate of about 20% to 35% at 10 mg/L and 100 mg/L cyprodinil after an initial rapid growth decline at 1 mg/L. Neither isolate was completely inhibited in mycelial growth at 100 mg/L iprodione, however, *C. acutatum* isolates were significantly more sensitive to that fungicide at concentrations of 10 mg/L and 100 mg/L (Fig. 1; Table 2).

3.2. OS-1 amino acid sequence analysis

The *OS-1* gene is a major component of the Hog1 MAPK pathway and mutations in that gene leading to amino acid substitutions have been linked to fludioxonil resistance (Ren et al., 2016; Sang et al., 2018; Zhou et al., 2020). The fragment sizes of the PCR products obtained were 2704 bp for *C. siamense*, 2704 bp for *C. fructicola*, 2547 bp for *C. nymphaeae,* and 2559 bp for *C. fioriniae*. A comparison of the OS-1 amino acid sequences between wildtype *B. cinerea* (GenBank; XP_024546375), *C. gloeosporioides* (UniProt; A0A010RRG8), and *C. fioriniae* of the *C. acutatum* complex (UniProt; T0JSQ2) revealed no amino acid changes in *Colletotrichum* spp. at codons previously linked to fludioxonil resistance in *B. cinerea* (Table 3). Except for A1259 and T1267, which were not part of the fragments amplified, the gene fragments obtained from our isolates confirmed the *Colletotrichum* spp. UniProt entries. For example, G545 of wildtype *B. cinerea* corresponded to the orthologous G545 locus in both *Colletotrichum* species complexes. Table 3 shows amino acid variations codons such as G545E and combinations such as G538R + A1259T reported to be linked to the fludioxonil high resistance (HR) phenotype. In all but two codons the *B. cinerea* wildtype allele (sensitive to fludioxonil) matched that of the orthologous codons of the *Colletotrichum* spp. complexes. Only *B. cinerea's* I365 and Q369 corresponded to V365 and K369 in *Colletotrichum* spp., respectively (Table 3). Because isolates of both species complexes had identical amino acid sequences at these two codons, they were not considered to be involved in the reduced sensitivity of *C. gloeosporioides* isolates to fludioxonil.

3.3. Osmotic sensitivity

Because resistance associated with the Hog1 MAPK pathway generally confers increased osmotic sensitivity (Li et al., 2020; Lin et al., 2018), the sensitivity of *Colletotrichum* spp. isolates to salt stress was investigated on 2%, 4%, and 6% NaCl media. Our results indicate that mycelial growth of *C. acutatum* isolates was not significantly more inhibited by increasing concentrations of NaCl compared to *C. gloeosporioides* isolates (Fig. 2).

3.4. Sensitivity of Colletotrichum spp. to cycloheximide

Cyclohexamide is an indicator of drug efflux pump activity in *B. cinerea* (Kretschmer et al., 2009). If a similar mechanism was active in *C. gloeosporioides* isolates, we would have expected their increased resistance to cycloheximide. Our study indicated, however, that *C. gloeosporioides* isolates were more sensitive to cycloheximide compared to *C. acutatum* isolates (Fig. 3).

4. Discussion

Multiple studies investigated the performance of Switch 62.5WG against *Colletotrichum* species of strawberry with varying disease management success. If *C. acutatum* was used to inoculate experimental plants, the fludioxonil + cyprodinil fungicide treatment was equal or superior in efficacy compared to other fungicide treatments including QoIs and captan (Haack et al., 2018; Daugovish et al., 2009; Mertely et al., 2010). In contrast, the same fungicide combination did not perform as well when plants were inoculated with *C. gloeosporioides*. Specifically, a 3 year study investigating strawberry plant mortality in plots treated with azoxystrobin, pyraclostrobin, thiophanate-methyl, and cyprodinil + fludioxonil showed that fludioxonil +_cyprodinil performed significantly worse than captan when applied protectively and significantly worse than QoI fungicides when applied curatively (MacKenzie et al., 2009). The results of our study suggest that the poor performance of the latter study may have been in part due to the *Colletotrichum* species used to inoculate the experimental plants.

The EC_{50} values reported for fludioxonil against *C. acutatum* isolates are consistently reported to be low indicating very high sensitivity. The mean EC_{50} values determined in mycelial growth assays for baseline *C. acutatum* isolates was 0.03 mg/L (Gao et al., 2018). The same study reports mycelia to be slightly more sensitive compared to germ tubes. Other studies also report EC_{50} values for fludioxonil against *C. acutatum* isolates to be <1 mg/L (Chechi et al., 2019b). Only one study reports

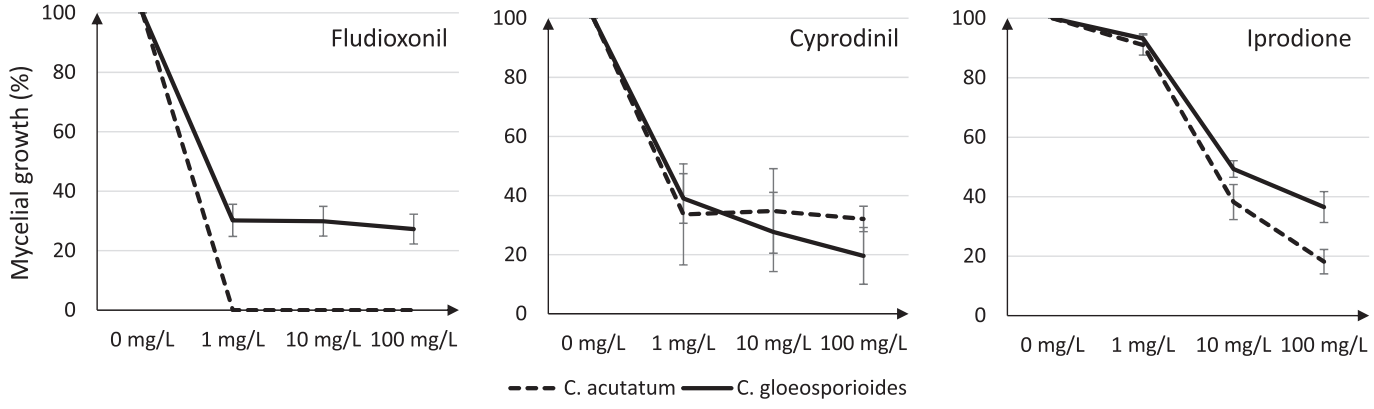

Fig. 1. Influence of increasing concentrations of fludioxonil, cyprodinil, and iprodione on mean mycelial growth displayed with 95% confidence interval of 6 *Colletotrichum acutatum* and 10 *C. gloeosporioides* isolates.

Table 2
Colletotrichum spp. minimum inhibitory concentration (MIC) values for fludioxonil, cyprodinil, and iprodione.

Species complex	Species	# isolates	MIC fludioxonil	MIC cyprodinil	MIC Iprodione
C. acutatum	*C. fioriniae*	3	≤1 mg/L	>100 mg/L	>100 mg/L
	C. nymphaeae	3	≤1 mg/L	>100 mg/L	>100 mg/L
C. gloeosporioides	*C. fructicola*	3	>100 mg/L	>100 mg/L	>100 mg/L
		1	>100 mg/L	>10 mg/L	>100 mg/L
	C. siamense	4	>100 mg/L	>100 mg/L	>100 mg/L
		2	>100 mg/L	>10 mg/L	>100 mg/L

Table 3
OS-1 amino acids in field or greenhouse isolates of *Botrytis cinerea* associated with fludioxonil high resistance (HR) phenotype and their orthologous amin acids from *C. fioriniae* PJ7 (UniProt; T0JSQ2), *C. gloeosporioides* Cg-14 (UniProt; A0A010RRG8) online sequences and partial gene fragments of *C. acutatum* and *C. gloeosporioides* isolates from this study.

Mutations in *B. cinerea* linked to fludioxonil resistance	Fludioxonil resistance phenotype	Citation	*C. acutatum*	*C. gloeosporioides*
F127S + I365N + S426N	HR	Sang et al., 2018	F127 + V365* + S426	F127 + V365* + S426
G538R + A1259T	HR	Sang et al., 2018	G538 + A1259	G538 + A1259
G545E	HR	Ren et al., 2016	G545	G545
N609T	HR	Ren et al., 2016	N609	N609
G265D	HR	Ren et al., 2016	G265	G265
G311R	HR	Ren et al., 2016	G311	G311
G262S + Q369P + N373S	HR	Ren et al., 2016	G262 + K369* + N373	G262 + K369* + N373
R319K + V336M + D337N + V346I + A350S + Q369P + N373S	HR	Ren et al., 2016	R319 + V336 + D337 + V346 + A350 + K369* + N373	R319 + V336 + D337 + V346 + A350 + K369* + N373
I365S + S531G + T565N + T1267A	HR	Zhou et al., 2020	V365 + S531 + T565 + T1267	V365 + S531 + T565 + T1267
I365S + S531G + T565N	HR	Zhou et al., 2020	V365 + S531 + T565	V365 + S531 + T565

* Amino acids in *C. siamense* and *C. fioriniae* different from *B. cinerea*.

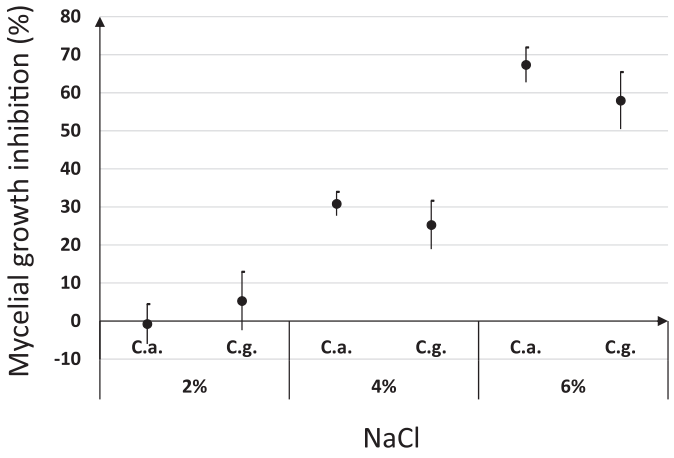

Fig. 2. Mean mycelial growth inhibition and 95% confidence intervals of 6 *Colletotrichum acutatum* (C.a.) and 10 *C. gloeosporioides* (C.g.) isolates on PDA amended with 2%, 4%, and 6% NaCl.

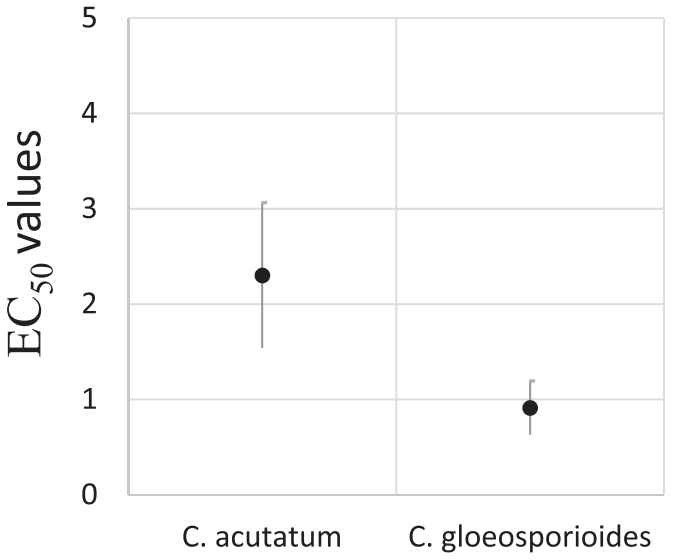

Fig. 3. Mean EC_{50} values for cycloheximide and 95% confidence intervals of 4 *C. acutatum* and 4 *C. gloeosporioides* isolates.

slightly higher EC_{50} values for *C. acutatum* isolates ranging from 0.3 to 1.5 mg/L (He et al., 2019). These data agree with our findings. The MIC values for our *C. acutatum* isolates were <1 mg/L and consequently the EC_{50} values (not calculated) were <1 mg/L. Although EC_{50} values <1 mg/L for *C. gloeosporioides* isolates were reported in some studies (Chechi et al., 2019; Khodadadi et al., 2020; He et al., 2019), this study shows that the calculation of EC_{50} values would be misleading. For example, *in vitro* sensitivity assessment of one *C. gloeosporioides* isolate revealed an EC_{50} of 2.87 mg/L but a MIC value of 7.3×10^3 mg/L (Lee, 2006). Our *C. gloeosporioides* isolates maintained mycelial growth after an initial 70% inhibition at 1 mg/L fludioxonil leading to MIC values >100 mg/L. Calculating EC_{50} values for these isolates would be highly subjective depending on what concentrations are involved in the calculation. In this study, a linear regression formed with only the lowest dose (1 mg/L) would have resulted in EC_{50} values <1 mg/L, but this would have been an inappropriate determination of EC_{50} values based on linear regression. A non-linear regression could be applied for better accuracy of EC_{50} determination but for all practical purposes the use of the value is irrelevant due to the high MIC values. An additional set of 12*C. gloeosporioides* isolates (4*C. siamense*, 4*C. fructicola*, and

4*C. theobromicola*) were investigated for sensitivity to fludioxonil *in vitro*. The results confirmed the strong initial inhibitory effect but also the lack of continued concentration response for *C. gloeosporioides* isolates (data not shown). Consequently, these isolates also had MIC values >100 mg/L.

Switch 62.5WG is a product with two active ingredients, fludioxonil and cyprodinil. Our study shows that even 100 mg/L cyprodinil was unable to completely inhibit mycelial growth of *C. acutatum* and *C. gloeosporioides* isolates. Strong initial inhibitory action but lack of continued concentration response was also reported when *C. acutatum* and *C. gloeosporioides* isolates were tested in microtiter assays at 3 μM and 30 μM cyprodinil (Abril et al., 2008; Smith et al., 2013). Most field trials have been conducted with the fludioxonil + cyprodinil mixture and rarely was this treatment compared to the solo products to separate efficacy contributions of either product. In one study, cyprodinil did not reduce severity or incidence of Glomerella leaf spot of apple caused by *Colletotrichum* species compared to the control (Kowata et al., 2010). The *in vitro* and in planta studies suggest that the activity of the fludioxonil + cyprodinil mixture against *Colletotrichum* species is mostly based on fludioxonil performance.

An investigation into possible mechanisms of intrinsic tolerance in *C. gloeosporioides* isolates to fludioxonil provided no indication of *OS-1* gene involvement. This investigation was initiated because *C. gloeosporioides* isolates were not only more tolerant to fludioxonil but also revealed increased tolerance to iprodione compared with *C. acutatum* isolates. Mutations in the *Botrytis* two-component osmosensing histidine-kinase gene (*Bos-1)* have been shown to confer high resistance to both fludioxonil and iprodione in previous studies (Ren et al., 2016; Sang et al., 2018; Zhou et al., 2020), but others have been identified as inconsequential (Grabke et al., 2014). Other studies document that the deletion of *CgHog1* and $CgPBS_2$ genes, both key components of the Hog1 MAPK pathway, resulted in enhanced sensitivity to osmotic stress and increased resistance to fludioxonil in *C. gloeosporioides* (Li et al., 2020; Lin et al., 2018). Similarly, *Neurospora crassa os-2* and *os-5* deletion mutants exhibited reduced sensitivity to fludioxonil indicating that multiple genes in the Hog1 MAPK pathway can be involved in fludioxonil resistance (Zhang et al., 2002). It is therefore possible that *C. gloeosporioides* isolates do possess one or more variations in other genes involved in the Hog1 MAPK pathway lending tolerance to fludioxonil. Except for one study (Avenot et al., 2005), increased sensitivity to salt stress has frequently been associated with fludioxonil-mediated alteration of the Hog1 MAPK pathway. Our results indicate that mycelial growth in *C. acutatum* isolates was not significantly more inhibited by increasing concentrations of NaCl compared to *C. gloeosporioides* isolates. In addition, we did not find specific alterations in OS-1 amino acid sequences between the two species complexes that were previously associated with HR in *B. cinerea* to fludioxonil (Ren et al., 2016: Sang et al., 2018; Zhou et al., 2020). There were, however, a few variations among the fludioxonil resistance-associated codons that were identical between the two *Colletotrichum* species complexes but different from *B. cinerea*, including V365 and K369 (orthologous to I365N and Q369P in *B. cinerea*, respectively).

Other mechanisms conferring resistance to fludioxonil in *B. cinerea* include ATP-binding cassette transporter and major facilitator superfamily transporter genes. The latter was shown to coincide with a significant (13-fold) increase in tolerance to cycloheximide (Kretschmer et al., 2009). However, our preliminary investigation did not show increased tolerance to cycloheximide in *C. gloeosporioides* isolates compared to *C. acutatum* isolates.

In conclusion, *C. acutatum* isolates and *C. gloeosporioides* isolates reveal significant differences in sensitivity to fludioxonil that may explain lack of field performance against strawberry crown rot and that should be taken into consideration for management of diseases caused by *Colletotrichum* spp.

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