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DEVELOPMENT OF MOLECULAR DETECTION SYSTEM FOR SDHI FUNGICIDE RESISTANCE AND FIELD ASSESSMENT OF SDHI FUNGICIDES ON SCLEROTINIA HOMOEOCARPA POPULATION INOCULATED WITH SDHI-RESISTANT ISOLATES

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A Dissertation Presented

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ABSTRACT

DEVELOPMENT OF MOLECULAR DETECTION SYSTEM FOR SDHI FUNGICIDE RESISTANCE AND FIELD ASSESSMENT OF SDHI FUNGICIDES ON *SCLEROTINIA HOMOEOCARPA* POPULATION INOCULATED WITH SDHI-RESISTANT ISOLATES

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Dollar spot, caused by the causal agent *Clarireedia* spp. (formerly, *Sclerotinia homoeocarpa*), is one of the most economically challenging turfgrass diseases in North America. To maintain acceptable quality of amenity turfgrasses, multiple fungicide applications are required. Since the launching of boscalid in 2003, succinate dehydrogenase inhibitors (SDHI) have been frequently used, becoming one of the most important fungicide classes not only for dollar spot control but for other plant pathogenic fungal diseases. However, repeated application of fungicides often lead to fungicide resistance. SDHI fungicide resistance has proven to be more complex than the resistance of other fungicide classes, with differential patterns of cross-resistance to five SDHI ingredients dependent on specific mutations to the succinate dehydrogenase (SDH) enzyme. Since 2016, our lab has received samples from several golf courses and one university research plot that experienced SDHI field failure against dollar spot. Through in vitro assays and DNA sequencing, our previous studies identified and profiled four mutations conferring differential SDHI sensitivity in *Clarireedia* spp.; an amino acid substitution H267Y and a silent mutation (CTT to CTC) at amino acid position 181 in

SDHB subunit, and amino acid substitution G91R and G150R in SDHC subunit. In this project, through in vitro assays and DNA sequencing, we identified and profiled two additional mutations conferring differential sensitivity; H267R in SDHB subunit and P80L in SDHC subunit. However, in vitro sensitivity assays alone can present numerous challenges and can sometimes provide inconclusive results. Therefore, in order to fully understand the complicated mechanisms of SDHI resistance, it is important to understand the association between in vitro assays and field efficacy. Further, the ability to quickly detect SDHI resistance using molecular tools could prove useful for providing fast and accurate recommendations for resistance management to practitioners. The first objective of this research was the development of a molecular detection system for SDHI resistance using molecular markers. Using cleaved amplified polymorphic sequence (CAPS) and derived CAPS (dCAPS) molecular markers, different types of mutations in SDHI-resistant isolates were clearly identified. The second objective was an investigation of the association between in vitro SDHI sensitivity and field efficacy. Following inoculation of turf research plots with the identified SDHI-resistant isolates, similar patterns of differential sensitivity that had already been profiled via in vitro assays were validated. In summary, it is important to monitor the distribution of resistance to SDHIs using both in vitro assay and molecular markers, to understand cross-resistance relationship among SDHIs including new chemistries to be registered, and to better understand the resistance mechanism for development of SDHI resistance management strategies.

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CHAPTER 1

DEVELOPMENT OF MOLECULAR MARKERS FOR SDHI RESISTANCE IN SDHI-RESISTANT FIELD ISOLATES *CLARIREEDIA* SPECIES

Abstract

Dollar spot, caused by an ascomycete fungus *Clarireedia* spp. (formally, *Sclerotinia homoeocarpa*) is one of the most resource-demanding diseases on amenity turfgrasses in North America. Since the launch of boscalid in 2003, succinate dehydrogenase inhibitors (SDHIs) have been frequently used for dollar spot control, and have become one of the most versatile and fast-growing fungicide classes. However, resistance to SDHIs has been recently reported in dollar spot as well as many other plant pathogenic fungal diseases. SDHIs have a complex mechanism of resistance, with different mutations on the succinate dehydrogenase (SDH) enzyme associated with differential sensitivity profiles to 5 SDHI active ingredients. Therefore, it is especially important to understand the complex mechanisms of SDHI resistance and develop molecular resistance detection systems, in order to provide fast and accurate recommendations for resistance management. Our previous study reported four mutations which confer differential sensitivities to SDHIs from *Clarireedia* spp. field isolates: an amino acid substitution H267Y; a silent mutation (CTT to CTC) at codon 181 in the *Sdh*B subunit gene; an amino acid substitution G91R; and an amino acid substitution G150R in the *Sdh*C subunit gene. In 2017, as part of this study additional SDHI-resistant *Clarireedia* spp. isolates were collected from Rutgers University research plots where repeated applications of boscalid selected for an amino acid substitution H267R at the

*Sdh*B subunit gene. In the present study, cleaved amplified polymorphic sequence (CAPS) and derived CAPS (dCAPS) molecular markers were developed to detect five mutations conferring SDHI resistance in *Clarireedia* spp. isolates and were validated using samples from additional two golf courses experiencing SDHI field failure. This molecular diagnostic tool will help develop strategies for dollar spot disease control and resistance management by monitoring of resistance in field populations.

Introduction

The ascomycete fungus *Clarireedia* spp. (formally known as *S. homoeocarpa*, Salgado-Salazar et al. 2018) is the causal agent of dollar spot. Characterized by dollar coin-sized bleaching appearance of the leaf blades dollar spot is the most economically important disease on cool-season turfgrasses in North America and worldwide (Smiley et al. 2005). Several fungicide classes are effective for the control of this pathogen but resistance to the methyl benzimidazole carbamate (MBC), dicarboximide, and demethylation inhibitor (DMI) fungicide classes have been reported across North America (Allan-Perkins et al. 2019; Bishop et al. 2008; Hulvey et al. 2012; Popko et al. 2012; Putman et al. 2010; Sang et al. 2015, 2016 and 2018). Since the launch of the active ingredient boscalid in 2003, the succinate dehydrogenase inhibitor (SDHI) class has become an important chemical strategy for turfgrass diseases control due to its broad spectrum of activity (Allan-Perkins et al. 2019, Klappach and Stammler 2019). Twenty SDHI active ingredients are listed by the Fungicide Resistance Action Committee (FRAC 2015). However, despite its relatively recent release, field resistance to the SDHI class has been reported in various plant pathogenic fungi (Sierotzki and Scalliet 2013) including *Clarireedia* sp. (Popko et al. 2018).

The active ingredients of SDHI fungicides suppress the process of cellular respiration by inhibiting the activity of complex II, a protein known as succinate dehydrogenase (SDH) in the electron transport chain (ETC). This protein consists of 4 subunits; a flavoprotein (SDHA), an iron-sulfur protein (SDHB), and two membrane-anchoring proteins (SDHC, SDHD). Subunit SDHB contains three iron-sulfur clusters and forms an ubiquinone (UQ) binding pocket with SDHC and SDHD (Hägerhäll 1997; Janssen et al. 1997). SDH inhibitors are able to bind these sites, thus inhibiting the process from succinate to fumarate in the Krebs cycle as well as the reduction of UQ (Klappach and Stammler 2019).

Mechanisms of SDHI resistance have been investigated as well as their mode of action and efficacy. The first case of resistance was reported 7 years after the introduction of carboxin, the first generation SDHI (Klappach and Stammler 2019). Resistance to the SDHIs generally occurs through target gene mutations on three of the SDH subunits; *SdhB*, *SdhC*, and *SdhD* as single nucleotide polymorphisms (SNPs), the most frequent form of amino acid substitution among alleles (Klappach and Stammler 2019; Rafalski 2002). There have been reports of many specific polymorphisms causing differential sensitivity profiles to SDHIs in many plant pathogenic fungal species. Interestingly, not all the mutations were formed at the site which is involved in UQ binding pocket (Sierotzki and Scalliet 2013). In *Clarireedia* spp., two *ShSdhB* mutations and two *ShSdhC* mutations as SNPs were confirmed as functional but the resistance mechanism was partially understood. (Popko et al. 2018).

The resistance to SDHIs is attributed to differential binding modes of each SDHI active ingredient. Unlike other single-site mode of action fungicide-resistance

mechanisms, different mutations that happened after repeated SDHI applications caused differential sensitivity to SDHIs (Klappach and Stammler 2019). Clearly different level of fungicide efficacy has led to questions toward the term ‘cross-resistance’, which means a phenomenon where a developed resistance to an active ingredient also becomes resistant to other active ingredients within the same class (Klappach and Stammler 2019). On contrary, some mutations have conferred increased sensitivity to some SDHIs (Sierotzki and Scalliet 2013). For example, in *Clarireedia* spp., an H267Y mutation in the *ShSdhB* gene is related to an increase in vitro sensitivity to fluopyram despite a significant decrease in sensitivity to other active ingredients in the same SDHI class (Popko et al. 2018).

Management of fungicide resistance is a very important issue for golf course manager as well as industries. Therefore, rising reports of resistance have led to molecular detection to be very important (Sierotzki et al. 2019). One of the molecular detection methods, which is called SNP genotyping, allowed detecting the SNPs conferring resistance. Many methods have been developed for SNP genotyping including restriction fragment length polymorphism (RFLP), allele-specific PCR, single-strand conformation polymorphism analysis, dideoxy fingerprinting, amplification refractory mutation system (ARMS), and other PCR-based molecular markers (Bostein et al. 1980; Mullis et al. 1986; Labrune et al. 1991; Newton et al. 1989; Sarkar et al. 1992; Southern 1975). Of them, PCR-based markers can be used for fast and reliable identification given the mutated gene has distinct polymorphism among alleles. Also, pyrosequencing, which is a quantitative detection method, is adapted for the development of molecular detection

system for *Botrytis cinerea* isolates which are resistant to SDHI fungicides (Gobeil-Richard et al. 2016).

The most frequently used PCR-based markers are cleaved amplified polymorphic sequences (CAPS) (Konieczny and Ausubel 1993) and derived CAPS (dCAPS), which utilize unique recognition sites of a specific restriction enzyme following amplification of the region where the mutation is positioned. In cases where no restriction sites are present, synthetic recognition sites can be incorporated into the target sequences through dCAPS, in which mismatching nucleotides are added to the primer used for amplification (Neff et al. 1998). This system has allowed for the development of an easier, more rapid, and reliable molecular system for diagnosing specific mutations than conventional in vitro sensitivity assays.

Since the in vitro sensitivity assay takes a long time to conduct and many samples are necessary particularly in field population monitoring studies, the development of molecular detection methods is important in the SDHI fungicide class due to many different mutations with differential sensitivity. Therefore, in this study, DNA sequences of SDH subunit genes B and C in *Clarireedia* spp. field SDHI-resistant and -sensitive isolates were compared. One CAPS and four dCAPS markers were developed to detect each SNP of five mutations that confer differential sensitivities to SDHIs. Furthermore, the markers were tested for validation using two golf course samples. The molecular markers will be useful for identifying the genotypes of *Clarireedia* spp. field isolates, to diagnose resistance to SDHIs at a molecular level and ultimately to allow developing management strategies of dollar spot through monitoring of resistance in field populations.

Materials and Methods

Fungal isolates

A total of 20 *Clarireedia* spp. field isolates were used in this study; five isolates (JTS30, J-5, J-19, M-1, and M-2) were selected as the representative of one sensitive (JTS30) and four resistant isolates harboring different mutations in SDH subunits. In vitro sensitivity profiles of the group where the representative isolates belong to were previously characterized (Popko et al. 2018), and were also assayed in this study for the purpose of comparison. As part of this study, sensitivity profiles of an additional fifteen (R99, R104, R130, R131, R161, R162, R163, R164, R177, R192, R214, R224, R223, R224, and R239) were characterized. These isolates were collected from Rutgers University (New Brunswick, NJ) research plots suspected to be SDHI resistance following repeated applications of boscalid (B. Clark, *personal communication*). For the validation of molecular marker usage, seven isolates (W-1, W-2, W-3, W-4, W-5, W-6, and W-7) randomly selected from the turf samples of two fairways in The Legend at Bristlecone (Hartland, WI), and five isolates (CT106, CT107, CT111, CT112, and CT302) randomly from turf samples of two tee boxes in Wethersfield Country Club (Wethersfield, CT) were used.

In vitro fungicide sensitivity assays

To assess the in vitro fungicide sensitivity of each isolate to SDHIs, 5 mm precultured mycelial plugs grown on potato dextrose agar (PDA) in a petri dish were placed on PDA and PDA amended with each of four SDHI commercial products; boscalid (Emerald 70WG, BASF), fluxapyroxad (Xzemplar 2.51SC, BASF), isofetamid

(Kabuto 3.3SC, PBI Gordon), and fluopyram (fluopyram 50SC, Bayer Crop Science), at a 1,000 μg ml^{-1} discriminatory concentration determined in the previous study (Popko et al. 2018).

Two perpendicular diameters of mycelial growth were measured by 16EX digital calipers (Mahr) following three days of incubation. Relative mycelium growth (RMG) was calculated by dividing each diameter of mycelium on fungicide-amended PDA by the diameter of mycelium on non-amended PDA. RMG values were converted to RMG% by multiplying 100 (Jo et al. 2006). Two replicate plates were used for one experiment, and a total of two experiments was repeated.

Statistical analysis

All isolates used in this study were classified according to mutation profiles. Analysis of Variance (ANOVA) and Tukey's honestly significant difference (HSD) test was conducted to separate mean RMG% values by each fungicide. All statistical analysis was performed by JMP (version 10.0; SAS Institute Inc.).

DNA extraction, polymerase chain reaction (PCR), and DNA sequencing

All of the isolates were grown on PDA for five days to produce enough mycelia for DNA extraction. Using sterile pestles, the mycelia from each isolate was collected from the surface of the media, and genomic DNA was extracted following the method from the previous study (Hulvey et al. 2012). All primers used for the amplification of each SDH subunit and for sequencing are presented in Table 1. PCR was carried out to a final volume of 25 μl, with 10 ng of fungal DNA, 200 μM of dNTPs, 0.5 μM of each

primer, 5 µl of 5X Q5 Reaction Buffer 5µl of 5X Q5 High GC Enhancer, and 0.25 µl of Q5 High-Fidelity DNA Polymerase (New England Biolabs). Using a Mastercycler® pro S (Eppendorf), PCR parameters were as follows: an initial denaturation at 98 °C for 30 sec, followed by 35 cycles; denaturation at 98 °C for 10 sec, annealing for 30 sec and elongation at 72 °C for 1 min, followed by final elongation at 72 °C for 2 min. Annealing temperatures vary depends on primer sequences and are listed in Table 1. Amplified fragments were purified by using DNA Clean & Concentrator (Zymo Research). Purified amplicons were sequenced by Psomagen (Cambridge, MA).

Molecular marker analysis

For the development of distinct markers for each mutation, DNA sequences of each of *ShSdhB, ShSdhC,* and *ShSdhD* genes were aligned and possible CAPS markers among the mutations were determined based on the presence of restriction sites. For the adequate primer design for CAPS analysis, annealing temperatures and GC percentage of each primer were considered. PCR conditions of the CAPS marker were the same as the PCR settings for DNA sequencing.

For the development of dCAPS markers, dCAPS finder 2.0 (Neff et al. 2002; http://helix.wustl.edu/dcaps/dcaps.html) was used to add appropriate mismatching nucleotides to each dCAPS primer, and also find available restriction enzymes. Insert detailed information on how did you select a specific nucleotides for the RE. PCR using dCAPS primers was performed with the same ingredients, by touchdown PCR program which consists of denaturation at 98 °C for 30 sec, and 8 touchdown cycles starting with 98 °C for 10 sec, annealing (annealing temperature of each primer +4 °C) for 30 sec,

elongation at 72 °C for 1 min, and 27 cycles (same condition to standard PCR described above but annealing temperature of each primer –4 °C), followed by final elongation at 72 °C for 10 min. Amplified fragments were purified by using DNA Clean & Concentrator (Zymo Research) and digested by each restriction enzyme for one hour. Final products were electrophoresed on 3% agarose gel. All primer sequences, annealing temperatures, and lengths of each product following restriction enzyme digestion are presented in Table 2.

Results

DNA sequence analysis of SDH subunit genes

The sequencing of SDH subunits genes of all isolates used in this study revealed target-site mutations on either *ShSdhB* or *ShSdhC* genes, and no mutations were found from the *ShSdhD* gene (Fig. 1). Total of five mutations were considered for a marker development. Briefly, in isolate J-5, an amino acid substitution from histidine to tyrosine at the amino acid position 267 (B-H267Y) was detected. At that same position, an amino acid substitution to arginine (B-H267R) was also found in isolates R99 through R231 (Fig. 1a). A silent mutation, an SNP from thymine to cytosine at the nucleotide position 596 (at the amino acid position 181 of Leucine) on the *ShSdhB* gene was confirmed from isolate M-2 (Fig. 1b). Also, two amino acid substitutions were detected at amino acid positions 91 and 150, where glycine was substituted with arginine (C-G91R and C-G150R), from isolates M-1 and J-10, respectively (Fig. 1b).

In vitro fungicide sensitivity assays of Clarireedia spp. *field isolates*

To investigate the resistance of *S. Clarireedia* spp. field isolates (JTS30, J-5, J-19, M-1, M-2 and R99 to R231), in vitro sensitivities to 4 SDHI active ingredients (boscalid, fluxapyroxad, isofetamid, and fluopyram) were assayed and presented as mean relative mycelial growth (RMG%) in Table 3. The Rutgers University isolates R99 to R231, which harbor the same mutations B-H267R were grouped together. Following statistical analysis, mean RMG% values for each active ingredient were compared among isolates.

The results from in vitro assays of reference isolates were basically consistent with the previous sensitivity profiling (Popko et al. 2018). Therefore, we presented results of previously untested Rutgers isolates here. Isolates R99 to R231 revealed significantly high resistance to boscalid at 67.69±2.75 RMG%, compared to isolate JTS30, which is a sensitive reference isolate. For fluxapyroxad, these Rutgers University isolates showed similar sensitivity levels as JTS30, J-5, and M-2 at 22.64±1.16 RMG%, and it was statistically lower than the values of isolates J-19 and M-1. Resistance to isofetamid of Rutgers University isolates was statistically not different from isolates J-5, M-1, and M-2, but compared to isolates JTS30 and J-19, the RMG% value was significantly higher than the value of isolate JTS30, and lower than the value of isolate J-19, at 43.63±1.16. For fluopyram, the RMG% value of Rutgers University isolates was statistically similar to the values of isolates JTS30 and M-1 at 43.38±1.18. But, isolate J-19 was more resistance, and isolates J-5 and M-2 were more sensitive to fluopyram than isolates R99 to R231.

Molecular marker analysis

Using DNA extracted from all isolates, PCR reactions were conducted by each primer set, and restriction enzyme digested each fragment (Table 2). For B-H267Y, PCR products were digested with the restriction enzyme Tsp45I. Only the amplicon of the mutant (B-H267Y) did not have any restriction enzyme cut sites, and thus, the mutant had one band while the other isolates had two products (Fig. 2). The length of each fragment is presented in Table 2. On this wise, dCAPS marker analysis was conducted for target mutations C-G91R and C-G150R by digestion with restriction enzymes SmaI and AvaII, respectively (Fig. 2). For B-H267R and B-L181, PCR products of the mutants contained the synthetic recognition sites of restriction enzymes (Hpy99I and BsmAI, respectively) while the PCR products of the wild-type did not. As a result, two fragments were observed for mutant (B-H267R and B-L181) PCR products following digestion with their respective restriction enzymes, whereas wild-type PCR products had only one fragment following digestion (Fig. 2).

Validation of the molecular marker usage

To demonstrate the validation of these molecular markers, randomly sampled seven isolates (W-1 to W-7) from Wisconsin golf course, and five isolates (CT106 to CT302) from Connecticut golf course were chosen for molecular marker analysis prior to sequencing their SDH subunit genes. After DNA extraction of the isolates, all the sets of molecular markers designed in this study were tested for the detection of mutations. All of the Wisconsin and Connecticut isolates contained B-L181 silent mutation-specific

marker, which was confirmed by sequencing (Fig. 3). The sequencing revealed an additional new amino acid substitution mutation, C-P80L only in the Wisconsin isolates.

Discussion

The current study reported the successfully development of five total CAPS and dCAPS markers for detecting each of five SNP mutations on the genes for *ShSdhB* or *ShSdhC*. As compared to other diagnostic methods such as genome sequencing, these PCR-based markers are more easy, rapid, and affordable for implementation by pathology labs. Further, as part of this study, *Clarireedia* spp. field isolates (R99-R239) from an additional location, Rutgers University, were characterized for differential sensitivity to SDHIs and subunit genes were sequenced to reveal an additional mutation on the ShSdhB subunit, B-H267Y. This mutation is at the same amino acid position as discovered in several isolates collected from Japan but with a different amino acid substitution conferring a different sensitivity profile.

Results of the marker development were validated in the fall of 2018 and 2019 after two additional isolates were received from golf courses in Wisconsin and Connecticut, as a known silent mutation, B-L181 was detected from both locations, as well as an additional mutation C-P80L. These results indicate the importance of sequencing additional dollar spot samples from many locations nationwide through multiple years' in order to monitor resistance and better understand the number of mutations that exist naturally in field populations. At present, this molecular detection system is useful for detecting known mutations but continuing to sequence the SDH

subunits of field isolates is necessary for further validation and detection of additional mutations.

The isolate M-2 is the first isolate from which a silent mutation at the amino acid position 181 of *ShSdhB* was detected. Interestingly, this mutation displayed resistance to boscalid and isofetamid, despite no amino acid substitution and was also detected from two additional golf course locations under different management regimes. Further investigation of this silent mutation is necessary to determine if the silent mutation is truly functional in resistance or if resistance is caused by an untargeted gene mutation. This amino acid position does not seem to be directly involved in the formation of a UQ pocket according to predictions of the mutated site (Popko et al. 2018). However, phenotypic or structural changes by silent mutations have been reported from human cancer cell studies (Sauna et al. 2007). Authors report that these unexpected structural changes may be due to codon usage. As mRNA is translated into amino acids, the primary structure is folded into complex proteins. Through this co-translational protein-folding, translational pauses are required for the protein to be folded ideally and this process should be subject to the mRNA codon (Kimchi-Sarfaty et al. 2007). Since organisms have frequent codons and rare codons, the folding of proteins under the direction of rare codons may result in slight structural changes as compared to the proteins folded under the direction of frequent codons at the same rate (Kimchi-Sarfaty et al. 2007). In addition, it is possible that an unknown mechanism may be involved, such as outside alteration of target genes. Yamashita and Fraaije (2018) reported non-target SDHI resistance, as an overexpression of an efflux transporter.

Histidine at the amino acid position 267 of *ShSdhB* (and its homologous position) is a highly important residue for conferring resistance to SDHIs as there are many reports of resistance in multiple plant pathogenic fungal species harboring mutations at this amino acid position, including *Clarireedia* spp. Through a genetic transformation system, the function of this amino acid position in resistance was confirmed (Popko et al. 2018). A substitution from histidine to arginine or histidine to tyrosine at this position was commonly reported across many plant pathogenic fungi (Sierotzki and Scalliet 2013). For example, in *B. cinerea*, the causal agent of gray mold, one of the most phylogenetically close fungal species to *Clarireedia* spp., had B-H272Y/R/L mutations (Sierotzki and Scalliet 2013). In *Alternaria alternata*, which mainly causes leaf spot in various crops, harbored B-H277Y/R mutations (Sierotzki and Scalliet 2013). This suggests that this histidine position is well conserved among multiple species. Further, the residue of this histidine may play a role in core-binding between SDHI active ingredients and the SDH protein. However, the effect of various amino acid substitutions at B-H267 on efficacy of SDHIs has not been well understood. In addition, previous monitoring studies with *B. cinerea* have shown that the H272R mutant (homologous to H267R in *Clarireedia* spp.) was the most frequent genotype in field boscalid-resistant populations of *B. cinerea* (Yin et al. 2011). Similarly, a study with *Mycosphaerella graminicola* showed H267Y was the most frequent mutation conferring carboxin resistance, which had been selected under repeated fungicide applications, while H267L accounted for only a small portion of the population (Scalliet et al. 2012). In our study, all isolates collected from Rutgers University research plots had the same mutation, B-H267R, following repetitive boscalid

applications. However, a B-H267Y mutation was detected in only some of the isolates sampled from the same area on a Japanese golf course.

The B-L181 mutation was detected multiple times across multiple locations where SDHI fungicides were applied, including one isolate from Rhode Island, four isolates from Wisconsin and 5 isolates from Connecticut. In order to better understand how active ingredients, select for specific mutations, population studies with more precise monitoring approaches are required.

To further understand the mechanisms behind SDHI resistance and develop cost-effective detection systems that do not require costly sequencing, collective efforts among academics, industries, and turfgrass managers should be initiated. Several studies regarding SDHI fungicide sensitivity should be launched immediately in order to stay ahead of resistance and are listed as follows: to understand the association between in vitro sensitivity of SDHIs on mutations and field efficacy, to validate the function of each mutation through genetic transformation and to correlate with the respiratory rate of each mutations, to understand how different mutations develop at a site, and to understand structure and dynamics of natural sensitive and resistant populations.

Table 1. Primers for amplification of each SDH subunit gene.

Primers name	Primer sequence (5'-3')	Target gene	Annealing temperatures (℃)
F_*ShSdhB*	ATGGCAGCTCTCCGCAAC	*ShSdhB*	67
R_*ShSdhB*	TTAAAAAGCCATCTCCTTCTTGATCTCC		
F_*ShSdhC*	CTTCCGCATCAACGACGATA	*ShSdhC*	66
R_*ShSdhC*	TCCTCTTGGGAGACCTCAT		
F_*ShSdhD*	TGATGAGTAGCCGAGCTAC	*ShSdhD*	64
R_*ShSdhD*	CTGCTCACATAATCTCGCTTTC		

Table 2. Primers and associated annealing temperatures and restriction enzymes for each dCAPS and CAPS analysis, and the sizes of products after restriction digestion. Derived nucleotides are highlighted in grey.

Primer name	Primer sequence (5'-3')	Target mutation	Restriction enzyme	Expected products (bp)	Annealing temp (℃)
F _B-H267Y	AGAAAAAGGAAGAACGAAAGGC	B-H267Y	Tsp45I	Wild-type : 54, 92 Mutant : 146	63
R _B-H267Y	TTAAAAAGCCATCTCCTTCTTGATC				
F _B-H267R	GACAACAGCATGAGCTTGTACAGACGTC	B-H267R	Hpy99I	Wild-type : 117 Mutant : 29, 88	68
R_ B-H267R	TTAAAAAGCCATCTCCTTCTTGATCTCCGCAATCGC				
F_ C-G91R	CCGCGCTAAACCGCATCCCG	C-G91R	SmaI	Wild-type : 19, 277 Mutant : 296	68
R_ C-G91R	AGCACTGGTCACACTCAACCCCACAAT				
F_ C-G150R	CGCATCCCAAGCCAAATGTCTCGGTC	C-G150R	AvaII	Wild-type : 23, 243 Mutant : 266	68
R_ C-G150R	CGCACCTCACCATCTACCAGCC				
F_ B-L181	TCAATTCTACAAACAGTACAAATCAATCAAGCCGTGTCT	B-L181	BsmAI	Wild-type : 186 Mutant : 41, 145	68
R_ B-L181	AGGTATTCTTCGCTGTTCCACCAGTACGAAGG				

Table 3. Mean relative mycelium growth percentage (RMG%) of *Clarireedia* spp. field isolates to four SDHI active ingredients.

Isolate	Mean RMG%[a]							
	Boscalid		**Fluxapyroxad**		**Isofetamid**		**Fluopyram**	
JTS30	18.28 ± 1.29	c [b]	21.73 ± 0.80	b	30.70 ± 0.79	c	44.21 ± 1.48	abc
J-5	60.38 ± 0.73	ab	21.32 ± 1.68	b	40.58 ± 0.70	bc	31.90 ± 1.70	d
J-19	69.41 ± 2.98	ab	51.17 ± 2.21	a	58.11 ± 2.15	a	52.77 ± 1.25	a
M-1	74.21 ± 3.16	a	54.04 ± 2.92	a	50.99 ± 2.92	ab	44.39 ± 1.67	abc
M-2	53.60 ± 1.50	b	19.83 ± 4.22	b	48.58 ± 4.22	ab	36.80 ± 1.55	cd
R99 to R231	67.69 ± 2.75	ab	22.64 ± 1.16	b	43.63 ± 1.16	b	43.38 ± 1.18	b
P value	***[c]		***		***		***	

[a] RMG% was calculated by dividing each mean diameter of isolate culture on PDA amended with each SDHI active ingredients by the mean diameter of isolate culture on non-amended PDA.
[b] Means with the same letter are not significantly different from each other, according to Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$).
[c] *** indicates p-value smaller than 0.001 ($p<0.001$).

a)

SdhB (JTS30) : KKEERKAALDNSMSLYRCHTILNCSRTCPKG 279
SdhB (J-5) : KKEERKAALDNSMSLYRCYTILNCSRTCPKG 279
SdhB (M-2) : KKEERKAALDNSMSLYRCHTILNCSRTCPKG 279
SdhB (R99) : KKEERKAALDNSMSLYRCRTILNCSRTCPKG 279

ShSdhB (JTS30): AAATCAATCAAGCCGTATCTTCAACACAAC 605
ShSdhB (J-5) : AAATCAATCAAGCCGTATCTTCAACACAAC 605
ShSdhB (M-2) : AAATCAATCAAGCCGTATCTCCAACACAAC 605
ShSdhB (R99) : AAATCAATCAAGCCGTATCTTCAACACAAC 605

b)

SdhC (JTS30) : YQPQVPWIMSALNRITGCILSGSFYVFGLTYL 106
SdhC (J-19) : YQPQVPWIMSALNRITGCILSGSFYVFGLTYL 106
SdhC (M-1) : YQPQVPWIMSALNRITRCILSGSFYVFGLTYL 106

SdhC (JTS30) : TFALPFTYHGFNGLRHLAWDAGKTFKNKEVI 168
SdhC (J-19) : TFALPFTYHGFNRLRHLAWDAGKTFKNKEVI 168
SdhC (M-1) : TFALPFTYHGFNGLRHLAWDAGKTFKNKEVI 168

Fig. 1. Sequence polymorphism between wild-type and mutant alleles of *ShSdhB* (a) or *ShSdhC* (b) genes. Isolates J-5 and R99 harbor mutation, which is originally histidine at the amino acid position 267. Isolate M-2 harbors silent mutation at amino acid position 181 (thymine to cytosine at the nucleotide position 596). Glycine was changed into arginine in M-1 (at the amino acid position 91) and in J-19 (at the amino acid position 150) isolates. The amino acid or nucleotide that does not match in the others is shown on a black background.

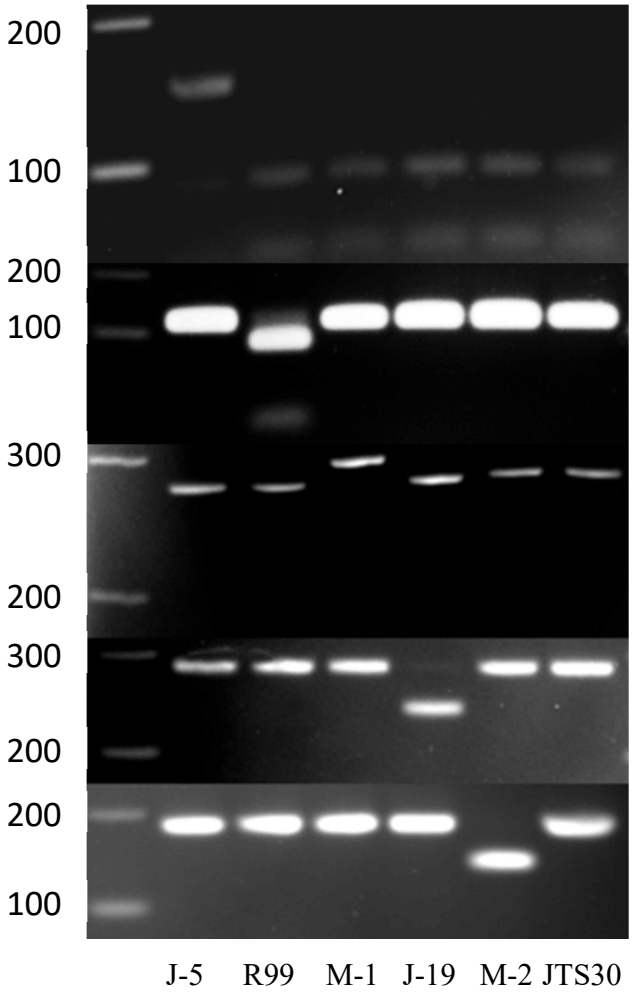


Fig. 2. Marker analysis of *ShSdhB* or *ShSdhC* gene mutations using representative isolates (J-5, M-1, J-19, M-2, and JTS30) and one of new isolates (R99). (a) CAPS analysis for detection of B-H267Y mutation using restriction enzyme Tsp45I, (b) dCAPS analysis for detection of B-H267R mutation using restriction enzyme Hpy99I, (c) dCAPS analysis for detection of C-G91R mutation using restriction enzyme SmaI, (d) dCAPS analysis for detection of C-G150R mutation using restriction enzyme AvaII, (e) dCAPS analysis for detection of B-L181 silent mutation using restriction enzyme BsmAI. All digested PCR products were electrophoresed on 3% agarose gel. The first lane is 100 bp DNA ladder (New England Biolab), and following lanes are reference isolates.

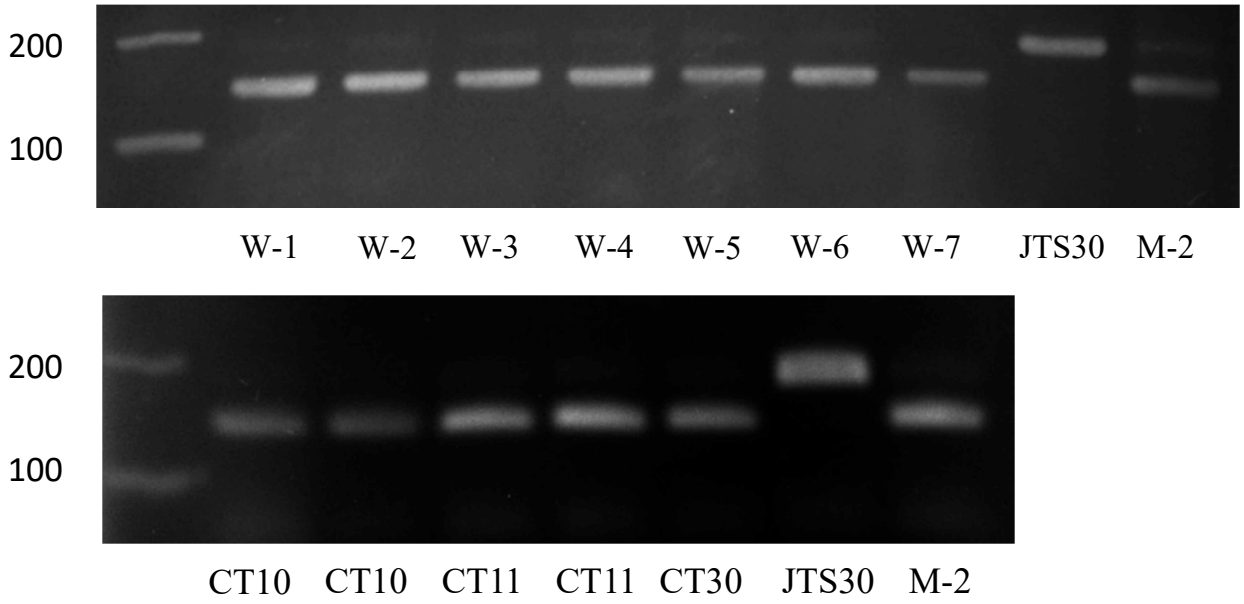


Fig. 3. Marker analysis of L181 silent mutation in *ShSdhB* gene using Wisconsin isolates (W-1 to W-7), Connecticut isolates (CT106 to CT302) and reference isolates (JTS30 and M-2). dCAPS analysis for detection of B-L181 silent mutation using restriction enzyme BsmAI. All digested PCR products were electrophoresed on 3% agarose gel. The first lane is 100 bp DNA ladder (New England Biolab), and following lanes are isolates for marker validation and reference isolates.

CHAPTER 2

FIELD ASSESSMENT OF SDHI FUNGICIDES ON *CLARIREEDIA* POPULATION INOCULATED WITH SDHI-RESISTANT ISOLATES

Abstract

Dollar spot, one of the most important major turfgrass diseases in North America, is caused by an ascomycete fungus *Clarireedia* spp. (formerly called *Sclerotinia homoeocarpa*). To maintain the quality of amenity turfgrasses, multiple classes of fungicides are used for dollar spot control. However, repeated fungicide application has caused reports of fungicide resistance, and SDHI fungicide resistance has been pointed out as more complicated than the resistance of other fungicide classes. Since 2016, the Turfgrass Pathology Lab at the University of Massachusetts Amherst has received reports of SDHI failure from several golf courses including University research plot. Previous studies profiled the mutations in the isolates collected from the locations; amino acid substitutions H267R/Y and a silent mutation (CTT to CTC) at amino acid position 181 in SDHB subunit, and amino acid substitution P80L, G91R, and G150R in SDHC subunit. In this study, field trials were conducted at three different locations over two dollar spot-seasons in 2018 and 2019, to evaluate the efficacy on different types of SDHI-resistant mutants. H267Y mutant had resistance to SDHIs but it was very sensitive to fluopyram. H267R mutant was highly resistant to boscalid. A mutant harboring the silent mutation B-L181 revealed resistance except pydiflumetofen. Mutations in SDHC subunit conferred overall resistance to SDHIs. The present study provides an understanding of resistance risk to SDHIs and gives the insight to monitor dollar spot populations.

Introduction

Clarireedia spp. (formerly, *Sclerotinia homoeocarpa*) is an ascomycete filamentous fungus that causes dollar spot, the most economically damaging disease of cool-season turfgrass in North America (Smiley et al. 2005). This disease can cause considerable damage to species in the family Poaceae, including annual bluegrass (*Poa annua* L.), colonial bentgrass (*Agrostis capillaris* L.), creeping bentgrass (*A. stolonifera* L.) and Kentucky bluegrass (*P. pratensis* L.), on golf course fairways, putting greens, and tee boxes (Latin 2011; Walsh et al. 1999).

Cultural practices often do not provide adequate dollar spot control. Therefore, multiple fungicide applications are required each year to maintain high turf quality (Smiley et al. 2005; Walsh et al. 1999). However, repeated fungicide applications on golf courses have led to the selection of *Clarireedia* sp. populations with resistance to the methyl benzimidazole carbamate (MBC), dicarboximide, demethylation inhibitor (DMI), and succinate dehydrogenase inhibitor (SDHI) fungicide classes (Allan-Perkins et al. 2017; Bishop et al. 2008; Cole et al. 1968; Detweiler et al. 1983; Golembiewski et al. 1995; Popko et al. 2018; Sang et al. 2015, 2016, and 2018).

As a fast-growing and relatively recently released chemistry, the SDHI fungicide class is an especially important penetrant class for dollar spot control (FRAC 2017; Sierotzki and Scalliet 2013; Allan-Perkins et al. 2019). Since the initial release of boscalid in 2003, five additional SDHI active ingredients have been registered for use on turf, including fluxapyroxad, penthiopyrad, isofetamid, fluopyram, and recently registered pydiflumetofen. Additional SDHI chemistries are currently in the registration

process. SDHI fungicides have a specific mode of action, targeting the cellular respiration of fungal pathogens. By binding a succinate dehydrogenase complex, which consists of four subunits (SDHA, SDHB, SDHC, and SDHD). SDHIs inhibit electron transfer from succinate to ubiquinone in the electron transport chain (ETC), leading to decreased energy production and arrested fungal growth (Matsson and Hederstedt 2001; Sierotzki and Scalliet 2013).

The Fungicide Resistance Action Committee (FRAC) categorizes the SDHI class as a medium to high risk for resistance development due to its highly specific mode of action (FRAC 2017). Therefore, resistance management practices are required to limit the development of SDHI resistance in *Clarireedia* sp. populations. However, mutations on the SDHB, SDHC, and SDHD subunits have already been reported to confer cross-resistance to SDHIs in multiple plant pathogenic fungi impacting many different crops (Sierotzki and Scalliet 2013). Each reported mutation confers a unique sensitivity profile to each SDHI active ingredient depending on the amino acid change and position (Klappach and Stammler 2019).

Recent studies have first reported mutations on *Clarireedia* spp. SDHB and SDHC subunits conferring differential sensitivity to active ingredients in the SDHI class (Popko et al. 2018). In brief, *Clarireedia* sp. isolates were collected from golf courses experiencing SDHI failure in Japan and Rhode Island. Following in vitro sensitivity assays and DNA sequencing, four different mutations across many isolates were identified to confer resistance. In the fall of 2018, we received samples from two additional locations experiencing SDHI failure, including a golf course in Wisconsin and research plots at Rutgers University in New Jersey. Therefore, another two mutations

were identified on the SDHB and SDHC subunits of these isolates, including C-P80L and B-H267R.

It is important to further understand the nature of each mutation to develop effective management strategies. However, it has not been reported how differently SDHI-resistant mutants behave in the field under the same environmental conditions. In this study, the isolates which have different SDH mutations were inoculated on the turf field at the University of Massachusetts, Amherst and SDHI fungicides were applied on a regular basis. This study was conducted over two dollar spot-seasons from different sites (putting green and fairways). The objectives of this study were (i) to evaluate field efficacy of the SDHI fungicides of *Clarireedia* sp. populations inoculated with wild-type and mutant strains, and (ii) to investigate the correlation between in vitro SDHI sensitivity and in field efficacy.

Materials and Methods

Clarireedia spp. field isolates

Eight isolates of *Clarireedia* spp. were used in this study. Name of the isolates, collected locations with the year, and their mutations are listed in Table 4. The isolates were maintained on potato dextrose agar (PDA) at 4°C until use. Artificial inoculums for inoculation were prepared by mixing 1 kg of autoclaved perennial ryegrass (*Lolium perenne*) seeds with mycelia grown on 4 PDA plates chopped into cubes, and 500 ml of potato dextrose broth (PDB). Subsequently, seeds with mycelia were incubated for seven days at room temperature.

Site selection and plot design

A field efficacy study was conducted at The Joseph Troll Turf Research Center (South Deerfield, MA). This study took place on putting green (*Agrostis palustris* 'Pure select') in 2018, and on two fairways (mixed stand of *A. stolonifera* 'Penncross' and *Poa annua*) in 2019. Prepared inoculums were distributed on the experimental plots on 6 June in 2018, 8 July (Location 1) and 14 August (Location 2) in 2019.

The plots were set up as a randomized complete block design with three replications. Each plot was 0.91 × 1.83 m in 2018, and 0.91 × 0.91 m in 2019. Treatments for field efficacy consisted of one untreated and five SDHI fungicides: boscalid (Emerald 70W, BASF; 0.18 oz/M), fluxapyroxad (Xzemplar 2.51SC, BASF; 0.26 oz/M), isofetamid (Kabuto 3.3SC, PBI Gordon; 0.5 oz/M), fluopyram (Fluopyram 50SC, Bayer Crop Science; 0.118 oz/M in 2018 and 0.15 oz/M in 2019), pydiflumetofen (Posterity 1.67SC, Syngenta; 0.16 oz/M). Fungicides were applied based on commercially recommended rates at a nozzle pressure of 40 psi by a CO_2-pressurized boom sprayer which is equipped with two flat-fan XR Teejet 8004VS nozzles. In 2018, all applications were made with 14 days intervals from 15 June to 10 August. In 2019, at Location 1, fungicides were sprayed from 11 July to 16 September with 14 days intervals except for the isofetamid treatment (21 days intervals). At Location 2, fungicide applications were conducted from 19 August to 2 October.

Disease evaluation

Dollar spot infection severity was estimated by percentages of dollar spot infected areas of each plot averagely every 6 days in 2018, and by a visual rating scale (1 = 0-10%,

2 = 10-20%, 3 = 30-50%, 4 = 60-80%, and 5 = 90-100%) of each plot averagely every 7 days in 2019. Subsequently, the evaluation was reported as the mean of three replications. The area under the disease progressive curve (AUDPC) values were calculated for the dollar spot percentage in 2018, and the dollar spot scale in 2019, using the formula: AUDPC = $\Sigma[(yi + yi + 1)/2](ti + 1 - ti)$, where $i = 1, 2, 3, \ldots, n - 1$ and yi is the amount of disease (disease percentage) at the time ti (days) of the ith rating (Campbell and Madden 1990). Mean separation on the disease severity was conducted for each rating date and the AUDPC to determine the effect of fungicide treatment on different isolates, using Tukey's highly significant difference (HSD) test (P = 0.05).

Results

The average area under the disease progress curve (AUDPC) values for each SDHI treatment are summarized for each inoculated *Clarireedia* spp. mutation population in Tables 5 and 6. Further, average disease values over tune for each isolate are presented in Figure 4, for visualization of the trend over the course of the study. AUDPC means were separated using Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$) and were used to indicate the efficacy of SDHI active ingredients on genotypically different mutants. Resistance patterns are reflected in the disease curves. As expected, all SDHIs were effective in reducing dollar spot infection on plots inoculated with non-mutated sensitive isolates, JTS30 in 2018 and HRS10 in 2019. Of all SDHIs tested, boscalid was the least effective among SDHIs with the highest AUDPC values, and pydiflumetofen was the most effective.

Isolates with B-H267R and B-H267Y mutations were both resistant to boscalid. However, the isolate harboring the B-H267Y mutation showed high resistance to boscalid from three different locations. On the fairways, resistance to fluxapyroxad, isofetamid, pydiflumetofen was also observed. However, this mutant was significantly susceptible to fluopyram with AUDPC values at 174 in 2018, 131.25 (Loc 1) and 127.67 (Loc 2) in 2019. The mutant B-H267R was also highly resistant to boscalid with AUDPC 307.33 and 264.33, and moderately resistant to pydiflumetofen with AUDPC 166.17 (Loc 1). However, it was relatively sensitive to fluxapyroxad, isofetamid, and fluopyram.

An isolate harboring a silent mutation B-L181, had resistance to boscalid, fluxapyroxad, isofetamid, and fluopyram. However, it was sensitive to pydiflumetofen with AUDPC 130.83 at Loc 2, which is significantly lower than other treatments. Similarly, the double mutant, which possesses the same silent mutation and C-P80L mutation, showed overall resistance to SDHIs except to pydiflumetofen, with the AUDPC value at 172.50.

The isolates possessing mutations in the SDHC subunit showed overall high resistance to SDHIs. The C-G91R mutant showed resistance to all SDHIs, but the levels of resistance of the isolate were different between treatments. This isolate is highly resistant to boscalid, but relatively less resistant to pydiflumetofen with the AUDPC values at 1351. The order of fungicide efficacy against this mutant was, pydiflumetofen > fluopyram = isofetamid > fluxapyroxad > boscalid. The isolate harboring C-G150R mutation showed overall high AUDPC values under SDHI treatments, which is unable to be statistically separated, from all three different locations. This suggests it has high levels of resistance to all SDHI active ingredients tested.

Discussion

To our knowledge this is a first report on field efficacy evaluation of SDHI fungicides on research turf green in 2018 and on two fairways in 2019 plots inoculated with *Clarireedia* spp. isolates harboring each of several SDH mutations on two of four SDH subunits, SDHB and SDHC. Our recent study indicated that mutations on the SDH subunits in *Clarireedia* spp. confer differential in vitro sensitivity to SDHI active ingredients (Popko et al. 2018). The present study validated differential sensitivities of each SDHI mutations to SDHI fungicides in a field setting. Results of both in vitro and field efficacy suggest that specific structural changes by unique SDH mutations can significantly affect the binding modes of SDHIs with the SDH complex, leading to resistance.

Mutations at the 267th amino acid histidine in SDHB have been frequently reported in multiple plant pathogens as substitutions to tyrosine, leucine, arginine, and valine. These mutations have been shown to be predominant in resistant pathogen populations, resulting in differential sensitivity to SDHIs in plant pathogenic bacteria (Li et al. 2006; Matsson and Hederstedt 2001) and fungi (Avenot et al. 2011, Landschoot et al. 2017; Shima et al. 2011; Veloukas et al. 2013). Previous structural analysis has shown that this conserved histidine residue is one of the components forming an ubiquinone binding pocket (Horsefield et al. 2006). The histidine residue at this position is also involved in hydrogen bonding with SDHI active ingredients, and is associated with the (3Fe-4S) high-potential nonheme iron sulfur-redox (S3) center (Skinner et al. 1998). Therefore, the replacement of histidine at this position will affect the binding mode of

SDHIs. A substitution to tyrosine (B-H267Y) conferred resistance to most SDHIs because the substitution impairs hydrogen bond to SDHIs (Scalliet et al 2012). On the other hand, the B-H267Y mutant had a sensitivity to fluopyram, which belongs to the benzamide derivatives. Since fluopyram does not include any hydrogen bond acceptor groups, a tyrosine substitution would not interrupt the ability of fluopyram to bind SDH complex (Scalliet et al. 2012). This high sensitivity to benzamide derivatives induced by this mutation at the homologous position has been already reported (Avenot et al. 2014; Gutiérrez-Alonso et al. 2017; Ishii et al. 2011; Scalliet et al 2012). Also, it was suggested that a tyrosine substitution will allow the benzamide structure to better bind the ubiquinone pocket (Popko et al. 2018). In contrast, the H267R mutant, in which the histidine is replaced by arginine, showed only high resistance to boscalid, and only somewhat decreased sensitivity to all other SDHIs. High resistance to boscalid by replacement from histidine to arginine at this position has been reported from for other plant pathogenic fungi, such as *Alternaria alternata* (Avenot et al. 2008), *A. solani* (Miles et al. 2014), *Botrytis cinerea* (Yin et al. 2011), and *Didymella bryoniae* (Avenot et al. 2011).

A mutant possessing a silent mutation at L181 (CTT to CTC) in SDHB, did not affect the amino acid sequences but clearly demonstrated field resistance to boscalid, fluxapyroxad, and isofetamid, and relative sensitivity to pydiflumetofen. Popko et al. (2018) showed the same mutant has in vitro resistance to boscalid, isofetamid, and penthiopyrad. Although silent mutations conferring resistance in fungal pathogens have not been reported, it was suggested that co-transcriptional protein folding can be affected by rare codons which may impact protein conformation (Kimchi-Sarfaty et al. 2007).

Also, as previous studies in *Clarireedia* spp. and *Z. tritici* reported non-target SDHI resistance as one of possible mechanisms, as altered expression of efflux pumps such as ATP-binding cassette (ABC) transporters and major facilitator superfamily (MFS) transporters (Sang et al. 2015; Yamashita and Fraaije 2018). The mechanism behind the isolate harboring silent mutation remains unclear.

Mutations that occurred on the SDHC subunit were not frequent compared to the SDHB mutations. This may be because SDHC and SDHD subunits are genetically less conserved than the SDHA and SDHB subunits. The substitution of glycine to arginine at codon 91 (G91R) in *Clarireedia* spp. conferred high resistance to boscalid, and moderate resistance to other SDHIs tested in this study. Increased fungicide resistance conferred by a substitution to arginine at the homologous position of G91 was also reported from *Pyrenophora teres* at C-G79R, and *Zymoseptoria tritici* at C-G90R (Rehfus et al. 2016 and 2017). This position has been already suggested to be involved in forming an α-helix out of five major helices of the SDHC subunit (Popko et al. 2018). The glycine at this position is also known to be close to heme b at the molecular level, and the substitution to arginine was suggested to induce spatial rearrangements that result in the failure of the positioning of the heme b molecule (Rehfus et al. 2016; Stammler et al. 2015). The C-G150R mutant showed the highest resistance to all SDHIs tested. Although any amino substitutions at this position or the homologous position have not been reported from other plant pathogenic fungi, the mechanism of resistance by G150R mutation seems to be similar to the mechanism by G91R because structurally the glycine at this position is involved in the interaction with heme b, as suggested for the G91R mutation.

Since the SDHI resistance becomes a significant problem for disease management, it has been investigated that the target mutation in SDH subunits is the key to resistance. Compared with previous studies of in vitro sensitivity by Popko et al. (2018), our study demonstrates a strong correlation between in vitro SDHI resistance and field efficacy on SDH mutants. One of the most interesting findings was that boscalid, the first active ingredient of the new generation of SDHI, provided no longer good control on dollar spot regardless of types of mutations tested here, but pydiflumetofen which is newly released was still effective to some of mutations. Especially fluopyram application can be a good suggestion to control dollar spot populations with H267R/Y mutations. However, frequent applications can induce the development of resistance caused by either non-target mutations or new different mutations on SDH subunit genes. This study could not explain how the SDHI-resistant isolates are selected and developed by specific SDHI fungicide treatments. However, we understand that it might be hard to predict different types of mutations to be developed due to many factors contributing development of SDHI resistance, such as their fitness cost. It is still in progress that how mutated SDH subunit gene affects rates of the cellular respiration, and how the SDHI active ingredients bind to the mutated SDH subunits in *Clarireedia* spp. Better understanding the mechanisms behind SDH mutations at the molecular level should be further investigated to develop environmental sustainable resistance management strategies.

Table 4. Fungal isolates of *Clarireedia* spp. used in this study.

Isolate no.	Location	Year collected	SDH subunit mutation[z]
HRS10	Hickory Ridge Golf Club, Amherst, MA	2012	…
JTS30	The Joseph Troll Turf Research Center, South Deerfield, MA	2012	…
J-15	Takehara Country Club, Hiroshima, Japan	2016	B-H267Y
J-19	Takehara Country Club, Hiroshima, Japan	2016	C-G150R
M-1	The Misquamicut Club, Westerly, RI	2018	C-G91R
M-2	The Misquamicut Club, Westerly, RI	2018	B-L181 silent
R99	Rutgers University, New Brunswick, NJ	2018	B-H267R
W-16	The Legend at Bristlecone, Hartland, WI	2019	B-L181 silent, C-P80L

[z] Mutations are named as follows: the subunit where mutation occurred, hyphen, the original amino acid, substituted amino acid position, and the substituted amino acid. In case of B-L181 silent mutation, amino acid was not substituted but only the 596th nucleotide of *ShSdhB* gene was changed from thiamine to cytosine.

Table 5. Area under the disease progress curve values for dollar spot mutants under SDHI fungicide treatments in 2018 on putting green.

	Mutation			
Fungicide	**Sensitive**	**B-H267Y**	**C-G91R**	**C-G150R**
		AUDPC[z]		
Untreated	1821 a[y]	1640 a	2383 a	1696
Emerald	327 b	1736 a	2371 a	1638
Xzemplar	251 b	755 b	2050 ab	1501
Kabuto	238 b	419 b	1573 bc	1310
Fluopyram	233 b	174 b	1675 bc	1663
Posterity	190 b	797 b	1351 c	1220

[z] Area Under the Disease Progress Curve.

[y] Means followed by the same letter are not significantly different according to Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$).

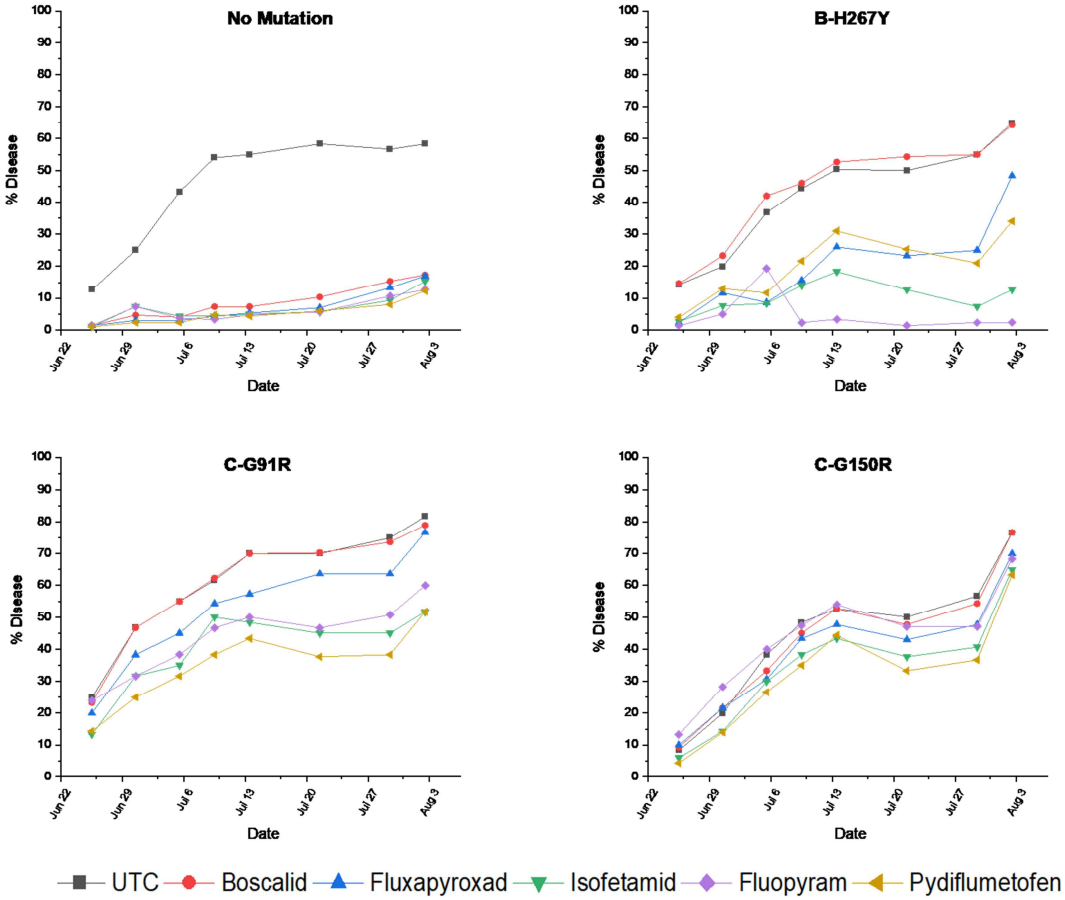
Table 6. Area under the disease progress curve values for dollar spot mutants under SDHI fungicide treatments in 2019 on fairways.

	Mutation					
Fungicide	**Sensitive**	**B-H267Y**	**B-H267R**	**B-L181 Silent**	**B-L181 Silent + C-P80L**	**C-G150R**
			AUDPC[z] at Location 1			
Untreated	228.67 a[y]	312.33 a	310.00 a	301.08 a	225.00	312.33
Emerald	141.83 b	315.00 a	307.33 a	306.42 a	228.25	304.33
Xzemplar	141.25 b	283.42 a	210.50 b	216.92 b	207.08	302.50
Kabuto	112.50 b	189.67 b	132.00 c	221.17 b	173.50	297.00
Fluopyram	113.17 b	131.25 b	126.67 c	271.75 a	229.33	297.67
			AUDPC at Location 2			
Untreated	215.33 a	264.17 a	259.33 a	264.17 a	267.00 a	267.00
Emerald	69.50 b	261.33 a	264.33 a	236.17 ab	260.50 a	253.17
Xzemplar	69.00 b	222.83 a	89.00 c	201.00 ab	235.00 a	263.50
Kabuto	75.83 b	219.50 a	79.67 c	231.50 ab	250.17 a	264.33
Fluopyram	76.33 b	127.67 b	63.33 c	187.83 bc	257.00 a	267.00
Posterity	58.67 b	243.50 a	166.17 b	130.83 c	172.50 b	258.83

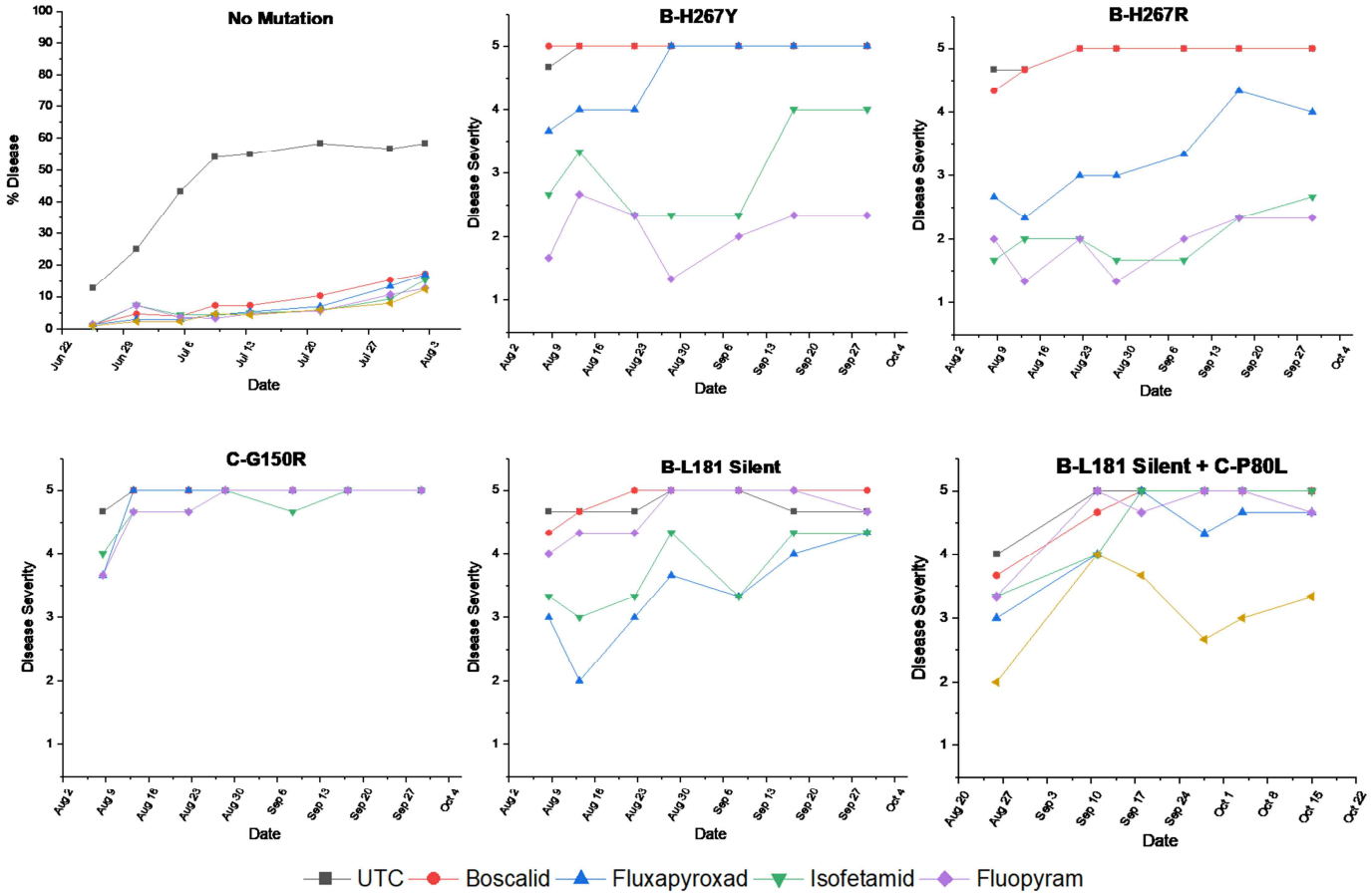
[z] Area Under the Disease Progress Curve.

[y] Means followed by the same letter are not significantly different according to Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$).

A


No Mutation
B-H267Y
C-G91R
C-G150R
% Disease
Date
Jun 22
Jun 29
Jul 6
Jul 13
Jul 20
Jul 27
Aug 3
UTC
Boscalid
Fluxapyroxad
Isofetamid
Fluopyram
Pydiflumetofen

B


No Mutation
% Disease
Date
B-H267Y
Disease Severity
Date
B-H267R
Disease Severity
Date
C-G150R
Disease Severity
Date
B-L181 Silent
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Boscalid
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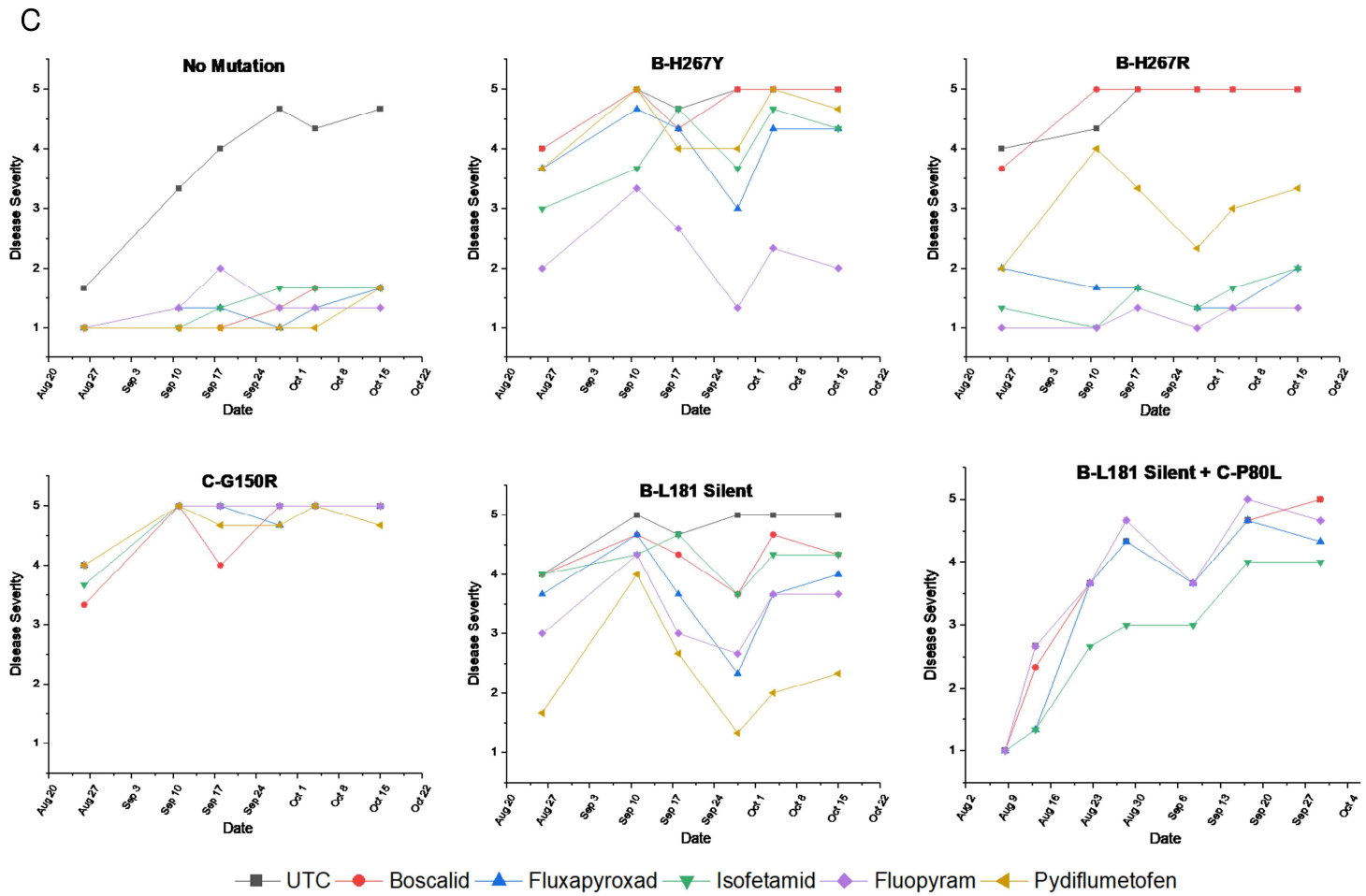

Fig. 4. Dollar spot disease progress curves under preventative application of SDHI active ingredients over time at (A) putting green in 2018, (B) fairway Location 1 in 2019, and (C) fairway Location 2 in 2019.

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