Distribution and Stability of Quinone Outside Inhibitor Fungicide Resistance in Populations of Potato Pathogenic *Alternaria* spp. in Wisconsin

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Abstract

Quinone outside inhibitor (QoI) fungicides have been an important class in managing potato early blight caused by *Alternaria solani* and brown spot caused by *A. alternata*. Because of the single-site mode of action character of QoI fungicides, which are relied on for management of diseases in Wisconsin, and the abundant asexual conidia production of the *Alternaria* species, pathogen isolates with QoI resistance have been detected after just a few years of QoI fungicide usage in commercial production fields. Resistance to QoIs has been attributed to amino acid substitutions F129L and G143A in cytochrome *b* of *A. solani* and *A. alternata*, respectively, as a result of point mutations. The aim of this study was to assess *Alternaria* populations in Wisconsin for QoI resistance before and after fungicide applications in order to evaluate resistance stability. A TaqMan single nucleotide polymorphism genotyping assay was designed based on the sequences of the cytochrome *b* gene from *Alternaria* isolates collected in Wisconsin to profile QoI resistance in *Alternaria* populations as well as to explore factors that may influence frequency of QoI resistance in the pathogen populations. This assay successfully identified the mutations conferring QoI resistance in isolates collected from four locations each year from 2015 to 2017. During the course of this study,

the frequency of *A. solani* isolates with the F129L mutation was consistently high and showed primarily the TTA mutation type. The frequency of *A. alternata* isolates with the G143A mutation started relatively low and increased at the end of the production season in each year ($P = 0.0109$, $P = 0.2083$, and $P = 0.0159$). A potato field managed without use of QoI fungicides showed a significantly lower ($P < 0.05$) frequency of *A. alternata* isolates carrying G143A than conventionally managed potato fields. The overall frequency of *A. alternata* isolates carrying G143A in the four locations was similar over the 3 years ($P = 0.2971$). The QoI resistance characteristics of the isolates were stable even when QoI selection pressure was removed for at least five sub-culture transfers, and the mutation types of codons 129 and 143 in the cytochrome *b* gene in *A. solani* and *A. alternata*, respectively, remained the same. This indicated that the application of QoIs in the field is not the sole factor responsible for the variation of the frequency of QoI resistance in the pathogen populations.

Keywords: fungicide resistance, potato early blight, potato brown spot, *Alternaria* spp.

Potato early blight, caused by *Alternaria solani*, and brown spot, caused by *A. alternata*, are two persistent potato foliar diseases that require routine fungicide applications for mitigation of yield and quality loss. Generally, brown spot is a relatively minor disease of potato compared with early blight. Foliar necrotic lesions and chlorosis caused by *Alternaria* spp. are common in Wisconsin and are usually first observed in early July and persist to harvest, if unmanaged. Reduced photosynthesis from necrosis can result in yield loss up to 30% annually in the case of early blight (Shtienberg et al. 1990). The pathogens have similar life cycles, as both are asexually propagated and cause polycyclic diseases with initial conidial inoculum dispersed by wind and rain splash (Agrios 2005; McCartney et al. 1993; van der Waals et al. 2003). Currently all predominant commercial potato varieties are susceptible to early blight and brown spot, and control is achieved with fungicides. In Wisconsin, a potato physiological day accumulation of 300 is used to guide the initial preventive fungicide application based on its validated association with an increase in conidia production (Pscheidt and Stevenson 1986). Because of the polycyclic nature of the diseases, subsequent fungicide applications are typically applied on a 7-day spray schedule

until vines are chemically desiccated at around 2 weeks prior to harvest.

Single-site mode of action fungicides have been used in managing early blight and brown spot since their registration in 1999, and fungicide resistance has been a progressive problem in many commercial fields (Pasche and Gudmestad 2008; Pasche et al. 2004; Rosenzweig et al. 2008a). One such single-site mode of action fungicide class is quinone outside inhibitors (QoIs), which have broad protection against both fungi and oomycetes and reduced negative effects on human health and the environment (Rosenzweig et al. 2008b; Tymon and Johnson 2014). By binding cytochrome *b*, QoI fungicides interrupt mitochondrial respiration to interfere with energy generation (Bartlett et al. 2002). Because they are highly effective in managing disease when the pathogen population is sensitive, QoI fungicides have been favored and used in many potato production regions (Pasche et al. 2004; Rosenzweig et al. 2008a). QoIs were initially released for early blight control, and they provided outstanding management of disease. However, because of its single-site mode of action, insensitive isolates within populations have been selected and prevailing insensitivity or resistance has accumulated quickly. Many studies have shown that single nucleotide mutations within the cytochrome *b* gene in targeted pathogens result in partial or complete resistance to QoIs (Ma and Michailides 2004; Pasche et al. 2004; Rosenzweig et al. 2008b). Several amino acid changes as a result of single nucleotide mutations may confer resistance, such as F129L, G137R, and G143A (Grasso et al. 2006). The F129L mutation, in particular, is known to confer partial resistance to QoI fungicides in *A. solani* (Bauske et al. 2018; Rosenzweig et al. 2008b). A G143A mutation in *A. alternata* confers complete resistance to QoI fungicides (Ma and Michailides 2004; Vega et al. 2012).

QoI resistance in *Alternaria* spp. has been reported in many regions. In the early 2000s, the majority of isolates from Wisconsin field populations demonstrated QoI resistance (Rosenzweig et al. 2008a), and the frequency was higher than 90% in Wisconsin after

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Funded: This project was partially funded by the University of Wisconsin-Madison Hatch Formula grant program.

*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary figures are published online.

Accepted for publication 17 March 2019.

2013 (Bauske et al. 2018). QoI resistance in *A. solani* has also been reported in Idaho, North Dakota, and Washington in the United States (Fairchild et al. 2013; Pasche et al. 2005; Tymon and Johnson 2014) as well as in other countries (Leiminger et al. 2014). QoI resistance in *A. alternata* has been reported in Idaho and Washington on potatoes (Fairchild et al. 2013; Tymon and Johnson 2014), and in Florida and California on other plant hosts (Karaoglanidis et al. 2011; Vega and Dewdney 2014). In Wisconsin and other potato producing regions, the reliance on QoI fungicides for the management of potato early blight and brown spot has decreased over the past 20 years owing to the reduced effectiveness of QoIs as a result of pathogen resistance in the field. Currently, QoI fungicides are still utilized in potatoes, but the targeted diseases are primarily below ground and include silver scurf, black dot, and Rhizoctonia root rot. Despite the general trend toward reduced foliar use of QoI fungicides, some growers still apply QoIs such as azoxystrobin (AZ) and pyraclostrobin, although not more than twice per growing season and usually mixed with a second fungicide of a different mode of action and in the early season for controlling early blight and brown spot together. QoI resistance in *A. alternata* has not been previously studied in Wisconsin and QoI resistance in *A. solani* has not been systematically studied in the state since 2002 (Rosenzweig et al. 2008b). With the change of fungicide programs, a contemporary assessment of QoI resistance in the field is needed to improve disease management recommendations.

The QoI resistance conferred by F129L in *A. solani* and G143A in *A. alternata* was not correlated with a fitness cost according to previous studies, indicating that the mitochondrial genotypes of the pathogens may be stable (Pasche and Gudmestad 2008; Vega and Dewdney 2014). The stability of phenotypic resistance and the efficacy of QoIs from year to year are not known. This information is very important to growers when they are making fungicide selection decisions. Therefore, the objectives of this research were to (i) assess *A. solani* and *A. alternata* populations in Wisconsin for QoI resistance by detecting the correlated mutations in cytochrome *b*, (ii) compare the frequency of resistant isolates before and after QoI application each season, and (iii) investigate the stability of QoI resistance by subjecting isolates to QoI selection pressure.

Materials and Methods

Sample collection. Sample collections were conducted in four potato fields in Plover, Hancock, Grand Marsh, and Madison, Wisconsin, each year from 2015 to 2017 (Table 1). The field located in Madison was organically managed and did not receive QoI fungicides, and the rest were commercial conventional fields. Each field was divided into five sections and from each section, five potato plants (at least 10 m away from each other) showing early blight and brown spot symptoms were arbitrarily selected for sampling. One symptomatic compound leaf was taken from each selected plant as one sample and all together 25 symptomatic compound leaf samples were taken from each field. Every field was visited twice, once before the foliar application of QoI fungicides (late June) and once in late August.

Isolation and identification. For each sample, lesion margins were excised from each compound leaf, surface sterilized with

70% ethanol for 30 s, and incubated on antibiotic-amended water agar (with 0.25% ampicillin and 0.05% rifampicin) to isolate the pathogens. Multiple lesions from each leaf were excised and cultured on individual Petri dishes, but only one single conidium from all resulting colonies with the same conidial morphology was picked and subcultured onto clarified antibiotic-amended V8 (CV8) juice agar (with 0.125% ampicillin and 0.05% rifampicin). To encourage sporulation, all cultures were incubated under 20 h of light and 4 h of darkness at 21 °C for 2 weeks. Based on conidia morphology, isolates producing long conidia (70 to 90 µm) with long beaks (30 to 40 µm) were putatively classified as *A. solani*. Isolates producing small conidia (25 to 35 µm) with short beaks (4 to 6 µm) were putatively classified as *A. alternata*.

For each *Alternaria* isolate, total DNA was extracted following the protocol of Bok and Keller (2012). The Alt a 1 gene was amplified using the primer pair Alt-for and Alt-rev (Hong et al. 2005). Amplification products were purified with the GenElute PCR Clean-Up Kit (Sigma-Aldrich) and sequenced at ProteinCT Biotech (Madison, WI). The sequences were aligned with reference sequences of *A. solani* and *A. alternata*, respectively, to corroborate the species identification.

Sequencing of cytochrome *b* (partial) and the TaqMan single nucleotide polymorphism genotyping assay. Amplification of cytochrome *b* from *A. alternata* was performed with primers DTRcytb2 (Grasso et al. 2006) and Aacytbr (5'-TGTAACCGTCTCCGTC-TATCAATC-3') at the annealing temperature of 52 °C for *A. alternata*. Aacytbr was designed based on the sequence of the GenBank accessions JQ446323 and JQ437357 (Vega et al. 2012). Amplification of cytochrome *b* from *A. solani* was performed with primers As5F and Asint129_r (Grasso et al. 2006) at the annealing temperature of 60 °C with High-Fidelity PCR polymerase following the manufacturer's recommended protocol (Clontech Laboratories Inc.). The above amplified segments were directly sequenced, and sequences were deposited in GenBank (accession numbers MH268171 and MH268172). Partial cytochrome *b* sequences of both species with mutations denoted were submitted to the Custom TaqMan Assay Design Tool (Thermo Fisher Scientific) for primer and probe designs to conduct the TaqMan single nucleotide polymorphism (SNP) genotyping assay (Table 2). The quantitative PCR (qPCR) was performed with total DNA of the isolates diluted to 10 ng/µl. Each qPCR reaction included 12.5 µl of 2× TaqMan Master Mix, 1.25 µl of 20× Assay Working Stock, 2 µl of diluted DNA, and 9.25 µl of Nuclease-Free Water (Thermo Fisher Scientific). The cycling conditions of qPCR included a single incubation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. The qPCR reactions were conducted using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories Inc.) and data were analyzed with Bio-Rad CFX Maestro software (version 4.0; Bio-Rad Laboratories Inc.).

QoI resistance stability test. Three isolates of *A. solani* and three isolates of *A. alternata* were chosen for a QoI resistance stability test based on the cytochrome *b* mutation types identified by the TaqMan SNP genotyping assay (Table 3). AZ, a common and representative QoI fungicide, was used to amend the media for this test. All of these isolates were grown on CV8 agar amended with AZ and CV8 agar without AZ continually for five subculture transfers with single

Table 1. Origin, number of applications of quinone outside inhibitor (QoI) fungicides, and collection year for *Alternaria solani* and *A. alternata* isolates characterized for the presence or absence of mutations associated with QoI resistance^z

Location	2015			2016			2017		
	QoI	Isolates		QoI	Isolates		QoI	Isolates	
		<i>A. solani</i>	<i>A. alternata</i>		<i>A. solani</i>	<i>A. alternata</i>		<i>A. solani</i>	<i>A. alternata</i>
Arena	2	16	21	1	43	31	1	25	47
Hancock	0	17	28	1	17	21	3	18	41
Plover	2	15	12	0	28	40	2	24	36
Madison	0	4	27	0	21	39	0	3	48
Total		52	88		109	131		70	172

^z One field from each location was studied every year from 2015 to 2017. QoI indicates number of times that a QoI fungicide was applied in each sampling year.

conidia. Each subculture transfer was grown for 7 or 10 days under the same conditions for sporulation. Mycelia were measured on day 10 postplate inoculation by measuring the radius of the growth for *A. solani* and on day 7 for *A. alternata*. Growth radii were averaged from two perpendicular measurements. After the measurement, the plates were flooded with water amended with Tween 20, and conidia suspensions were collected and adjusted to 5,000 conidia/ml. Then 10 µl of the conidia suspension was transferred to plates of water agar containing 0, 0.001, 0.01, 0.1, 1, and 10 mg/liter of AZ and 100 mg/liter salicylhydroxamic acid (SHAM). SHAM was used to inhibit alternative oxidase pathways that are potentially utilized by AZ-resistant *Alternaria* isolates (Fan et al. 2015; Rosenzweig et al. 2008a; Tymon and Johnson 2014; Ziogas et al. 1997), and we confirmed that 100 mg/liter of SHAM had no deleterious effects on conidia germination of the selected isolates following Pasche et al. (2004). After incubation for 4 h under ambient conditions on a laboratory bench top, the germinated and nongerminated conidia were counted. The EC₅₀ value of each subculture was calculated with the germination rate following the method described by Rosenzweig et al. (2008a). Using the same culture plates, the conidia and mycelia were collected for DNA extraction and subsequent TaqMan assays to detect the stability of the genotype of the cytochrome *b* genes over the serial transfers.

Statistical analysis. The overall frequency of *Alternaria* isolates carrying the mutations conferring QoI resistance was compared across the four locations using the Tukey test in SAS (SAS Institute). The frequency of QoI resistance from each year was compared using the Pearson χ^2 test in R software (version 3.4.2; R Core Team) for each location and overall to test whether the frequency changed from 2015 to 2017.

The ratio of QoI resistance from each location from early season to late season was compared with a *t* test, and the cutoff to determine significant differences was set at $\alpha = 0.05$.

Mycelial growth of each isolate was compared among subcultures 1, 3, and 5 grown on 1 mg/liter of AZ plus SHAM and on CV8 using Proc GLM analysis in SAS, and significant differences were determined at the $\alpha = 0.05$ level using the Tukey test. EC₅₀ values of the

isolates were compared using the same analysis method as the growth rate.

Results

Isolation and identification. Based on morphological characteristics and Alt a 1 sequencing results, 52, 109, and 70 isolates of *A. solani* were retrieved from 2015, 2016, and 2017, respectively. A total of 88, 131, and 172 isolates of *A. alternata* were retrieved from 2015, 2016, and 2017, respectively (Table 1). The cytochrome *b* sequence of *A. solani* was genotype II according to Leiminger et al. (2014). The cytochrome *b* sequence of *A. alternata* was the same as the *A. alternata* cytb profile I documented by Vega et al. (2012).

Development of primers and probes. Our results indicated that the TaqMan SNP genotyping assay was successful in discriminating different genotypes of codon 129 in *A. solani* and codon 143 in *A. alternata*. Therefore, the TaqMan SNP genotyping assays designed in this study were adequate to identify the mutation types at amino acid position 129 in cytochrome *b* of *A. solani* by pairing the probes identifying TTG and TTA and the probes identifying CTC and TTC. The probes designed for *A. alternata* also successfully detected the presence of mutations at amino acid position 143 in cytochrome *b*. All of the *A. solani* isolates carried the wild-type allele TTC at codon 129 (Supplementary Fig. S1). Most isolates also carried the mutated allele TTA, indicating mixed mitochondrial genomes (Supplementary Fig. S2). In addition, a few *A. solani* isolates showed mixed mutation genotypes at codon 129. However, most of the isolates for *A. alternata* carried either the wild-type allele GGT at codon 143 or the mutated allele GCT (Supplementary Fig. S3).

Determination of spatiotemporal distribution frequency of mutations conferring QoI resistance in *A. solani*. All of the *A. solani* isolates collected from 2015 to 2017 carried the mutations at amino acid position 129 in the cytochrome *b* gene. Among the mutant types, TTA was most common, followed by TTG and CTC, in 2015 and 2017. TTA was also the most common type, followed by CTC and TTG, in 2016. Most of the isolates showed only one type of mutation in cytochrome *b*, but two types of mutations were observed in several isolates (Table 4).

Table 2. Primers and fluorescent-labeled probes used in the quantitative PCR assay in detecting single nucleotide polymorphisms in the cytochrome *b* gene in *Alternaria solani* and *A. alternata*

Primer/probe	Sequence (5' to 3')
<i>A. solani</i>	
129 forward primer	AGAACTCTAGTATGAAGTATTGGTACTGTTATC
129 reverse primer	CAAACCATTTTGGGCTATGTTGGT
129 mutant (TTG)	[VIC] CCCAGCAAAGCTG
129 mutant (TTA)	[FAM] AACCCAGTAAAGCTG
129 wild type (TTC)	[VIC] AACCCAGGAAAGCTGT
129 mutant (CTC)	[FAM] CCCAGGAGAGCTGT
<i>A. alternata</i>	
143 forward primer	TTCTTGGGATACGTCTTGCCATAC
143 reverse primer	TTGACCTACTCAAGGTATAGCACTCA
143 mutant (GCT)	[VIC] TGTCATTATGAGCTGCAACA
143 wild type (GGT)	[FAM] ATGTCATTATGAGGTGCAACA

Table 3. Isolate number, year of collection, collection location, and type of cytochrome *b* detected in *Alternaria solani* and *A. alternata* used in the quinone outside inhibitor (QoI) resistance stability test

Species	Isolate no.	Year	Location	Initial		After subculturing on azoxystrobin	
				Genotype of cytochrome <i>b</i> ^y	Sensitivity to QoI ^z	Genotype of cytochrome <i>b</i>	Sensitivity to QoI
<i>A. solani</i>	As1	2016	Arena	TTC, CTC	Resistant	TTC, CTC	Resistant
	As2	2016	Madison	TTC, TTA, CTC	Resistant	TTC, TTA, CTC	Resistant
	As3	2016	Arena	TTC, TTA, TTG	Resistant	TTC, TTA, TTG	Resistant
<i>A. alternata</i>	Aa1	2017	Plover	GGT	Sensitive	GGT, GCT	Resistant
	Aa2	2017	Plover	GGT, GCT	Resistant	GGT, GCT	Resistant
	Aa3	2017	Plover	GGT, GCT	Resistant	GGT, GCT	Resistant

^y For *A. solani*, mutations were in amino acid 129 in the cytochrome *b* gene; for *A. alternata*, the mutation was in amino acid 143 in the cytochrome *b* gene.

^z *Alternaria solani* with F129L and *A. alternata* with G143A were considered as QoI resistant.

Determination of spatiotemporal distribution frequency of mutations conferring QoI resistance in *A. alternata*. Unlike *A. solani*, only a portion of the *A. alternata* isolates carried the mutations conferring QoI resistance, and that portion showed a spatiotemporal dynamic. The overall percentage of *A. alternata* isolates carrying the G143A mutation was 56, 45, and 51% in 2015, 2016, and 2017, respectively (Table 5). Despite different fungicide programs and the number of QoI applications used in fields, the frequency of *A. alternata* isolates carrying G143A mutations from the commercial fields over the 3 years was similar ($\chi^2 = 2.4$, $df = 2$, $P = 0.2971$) according to the Pearson χ^2 test (Table 5). However, the frequency of QoI

resistance in *A. alternata* changed over the 3 years in Arena ($P < 0.01$), Hancock ($P < 0.01$), and Madison ($P < 0.01$) but remained the same in Plover ($P = 0.2740$). The frequency of *A. alternata* isolates carrying G143A mutations from the Madison organic fields was significantly lower than that in Arena and moderately lower than Hancock and Plover based on results of a Tukey test (Table 5). From early to late season (Table 6), the frequency of QoI resistance in *A. alternata* increased in 2015 ($P = 0.0109$) and 2017 ($P = 0.0159$) but stayed the same in 2016 ($P = 0.2083$).

Growth rate and EC₅₀ stability with QoI selection pressure. In general, mycelial growth and EC₅₀ of the selected *A. solani* isolates

Table 4. Collection year, location, time, number of isolates, number of isolates with wild-type cytochrome *b*, and number of isolates with amino acid 129 mutations in cytochrome *b* of *Alternaria solani*

Year	Location	Time	No. of isolates	Wild type			Mutant type			
				TTC	TTA	(%)	TTG	(%)	CTC	(%)
2015	Arena	June	3	0	3	100	0	0	0	0
		August	13	0	11	85	1	8	1	8
	Hancock	June	9	0	9	100	0	0	0	0
		August	8	0	8	100	0	0	0	0
	Plover	June	15	0	14	93	1	7	0	0
		August ^t								
2016	Madison	June	0	0	0	0	0	0	0	0
		August	4	0	4	100	0	0	0	0
	Arena	June ^u	20	0	19	95	2	10	1	5
		August ^v	22	0	22	100	3	14	0	0
	Hancock	June	1	0	1	100	0	0	0	0
		August	16	0	16	100	0	0	0	0
2017	Plover	June	9	0	9	100	0	0	0	0
		August	19	0	19	100	0	0	0	0
	Madison	June	0	0	0	0	0	0	0	0
		August ^w	21	0	20	95	0	0	7	33
	Arena	June	0	0	0	0	0	0	0	0
		August ^x	25	0	25	100	1	4	0	0
2017	Hancock	June	0	0	0	0	0	0	0	0
		August ^y	18	0	18	100	1	6	0	0
	Plover	June	0	0	0	0	0	0	0	0
		August ^z	24	0	23	96	1	4	1	4
	Madison	June	0	0	0	0	0	0	0	0
		August	3	0	3	100	0	0	0	0

^t Lack of data as a result of early vine kill and harvest of the potato crop.

^u Two isolates had TTA and TTG, and one isolate had CTC.

^v Three isolates had TTA and TTG.

^w Six isolates had TTA and CTC, and one isolate had CTC.

^x One isolate had TTA and TTG.

^y One isolate had TTA and TTG.

^z One isolate had TTA and TTG, and one isolate had CTC.

Table 5. Number of *Alternaria alternata* isolates, number of *A. alternata* isolates that carried the G143A mutation conferring resistance to quinone outside inhibitors (QoI) collected from each field during 2015 to 2017, and frequency of *A. alternata* isolates that carried mutations

Year	No. of isolates	Location				Total
		Arena	Hancock	Plover	Madison	
2015	Total	21	28	12	27	88
	Carried mutation	19	23	5	2	49
	Frequency (%)	90	82	42	7	56
2016	Total	31	21	40	39	131
	Carried mutation	20	8	21	10	59
	Frequency (%)	65	38	53	26	45
2017	Total	47	41	36	48	172
	Carried mutation	26	25	18	19	88
	Frequency (%)	55	61	50	40	51
Mean frequency (%) ^x		70.1 ± 10.5	60.4 ± 12.7	48.1 ± 3.3	24.2 ± 9.3	
Tukey test $P < 0.05$ ^y		a	ab	ab	b	
Pearson χ^2 test ^z		$\chi^2_2 = 31.0$, $P < 0.01$	$\chi^2_2 = 16.9$, $P < 0.01$	$\chi^2_2 = 2.6$, $P = 0.2740$	$\chi^2_2 = 29.8$, $P < 0.01$	$\chi^2_2 = 2.4$, $P = 0.2971$

^x Mean frequency at each location was the average of frequency of G143A-mutant *A. alternata* isolates collected in 2015 to 2017 from each location.

^y Comparison of mean frequencies was conducted with a Tukey test and the significant difference was based on a P value of 0.05.

^z A Pearson χ^2 test was conducted to test whether the frequency of G143A-mutant *A. alternata* isolates collected from each location changed over the years.

increased with additional subculturing on CV8 amended with AZ, and these values remained the same when subcultured on CV8 (Fig. 1; Table 7). From the first transfer to the fifth transfer, mycelial growth of *A. solani* isolates As1 and As3 increased when grown only on AZ-amended media, but the EC₅₀ remained unchanged (Fig. 1; Table 7). However, for *A. solani* isolate As2, both EC₅₀ and mycelial growth values increased ($P < 0.05$ for both) from the first subculture to the fifth when constantly grown on AZ-amended media (Fig. 1; Table 7).

For the selected *A. alternata* isolates, mycelial growth of isolate Aa1 increased from the first subculture to the fifth, but the growth

Table 6. Collection year, location, time, number of isolates, number of isolates with wild-type cytochrome *b*, and number of isolates with amino acid 143 mutations in cytochrome *b* of *Alternaria alternata*

Year	Location	Time	No. of isolates	Wild type ^v		Mutant type	
				GGT	GCT	(%)	
2015 ^w	Arena	June	4	1	3	75	
		August	17	1	16	94	
	Hancock	June	12	3	9	75	
		August	16	2	14	88	
	Plover	June	12	7	5	42	
		August ^x					
	Madison	June	11	11	0	0	
		August	16	14	2	13	
2016 ^y	Arena	June	9	4	5	56	
		August	22	7	15	68	
	Hancock	June	10	5	5	50	
		August	11	8	3	27	
	Plover	June	16	12	4	25	
		August	24	7	17	71	
	Madison	June	22	18	4	18	
		August	17	11	6	35	
2017 ^z	Arena	June	26	18	8	31	
		August	21	3	18	86	
	Hancock	June	23	15	8	35	
		August	18	1	17	94	
	Plover	June	19	11	8	42	
		August	17	7	10	59	
	Madison	June	26	19	7	27	
		August	22	10	12	55	

^v Wild-type isolates do not have the G143A mutation.

^w Resistance % (August) > resistance % (June), $t = 6.667$, $df = 2$, $P = 0.0109$.

^x Lack of data as a result of early vine kill and harvest.

^y Resistance % (August) > resistance % (June), $t = 0.9398$, $df = 3$, $P = 0.2083$.

^z Resistance % (August) > resistance % (June), $t = 3.811$, $df = 3$, $P = 0.0159$.

of isolates Aa2 and Aa3 remained unchanged (Fig. 2). The EC₅₀ of isolate Aa1 and Aa3 increased with additional subculturing on CV8 amended with AZ (Table 8). The EC₅₀ of isolate Aa2 exceeded 10 mg/liter for subcultures grown on CV8 and on AZ-amended CV8. The EC₅₀ value of the third and fifth transfers of isolate Aa3 on AZ-amended media also exceeded 10 mg/liter starting from the third transfer (Table 8).

The TaqMan genotype assay revealed the same codon types of 129 in the cytochrome *b* gene in *A. solani* isolates and 143 in the cytochrome *b* gene in *A. alternata* isolates after serial culturing on CV8 media or AZ-amended media, with the exception of *A. alternata* isolate Aa1 (Table 3). The mutated codon type of GCT was detected in the third and fifth transfers of *A. alternata* isolate Aa1 on media amended with AZ (Table 3).

Discussion

This study documented the presence of QoI resistance in both *A. solani* as well as *A. alternata* from several potato production locations in Wisconsin. We found a consistently high, season-long frequency of *A. solani* isolates carrying QoI resistant mutations and a trend of increasing frequency of *A. alternata* isolates carrying QoI resistance from early season to late production season. Such resistance could be selected rapidly, within just one culture transfer on media amended with AZ, a common, representative QoI active ingredient. The resistance was stable for at least five culture transfers on nonselective media.

The cytochrome *b* TaqMan assay used in this study detected mutations in the cytochrome *b* gene in both *Alternaria* species, indicating that all of the *A. solani* isolates collected were genotype II or “U.S. genotype” as defined by Leiminger et al. (2014). Both genotypes I and II were discovered in Germany; however, the distribution of the two genotypes in the United States is unclear (Bauske et al. 2018). With the isolates collected in this study, it is likely that genotype II is the predominant, if not exclusive, genotype in Wisconsin.

Wild-type codon 129 in the cytochrome *b* gene was detected in all of the *A. solani* isolates, indicating that there was a significant genetic shift attributable to QoI selection pressure. Because *A. solani* is a specific pathogen on potatoes and tomatoes, the pathogen population has been under QoI selection pressure routinely since 1999 (the year in which AZ was registered for use on potato). As a consequence of such constant selection pressure, the resistant isolates may have become the natural population, with mutations now fixed in most field-collected resistant isolates (Van Leeuwen et al. 2008). Even in the organically managed field in Madison, where QoI fungicides were not applied, the *A. solani* isolates showed mutations linked to QoI resistance. We do not discount the possibility that the initial inoculum of these isolates could have come from infected seed potatoes

Fig. 1. Comparison of *Alternaria solani* mycelial growth at day 10 when transferred and grown on clarified V8 juice (CV8) amended with 1 mg/liter of azoxystrobin (AZ) and 100 mg/liter of salicylhydroxamic acid (SHAM) (shown as black columns) and CV8 without AZ and SHAM (shown as gray columns) one, three, and five times. Different letters above the columns indicate significant differences at $\alpha = 0.05$ based on the Tukey test.

(Secor and Gudmestad 1999) or been windblown from other conventional fields in which QoIs have been applied.

Different cytochrome *b* mutation types were present in *A. solani*, although TTA was the most prevalent for codon 129. This is consistent with a previous study by Rosenzweig et al. (2008a) which investigated isolates collected in 2001 to 2003 in Wisconsin. In their study, no

significant differences in sensitivity to AZ were observed among *A. solani* isolates carrying different types of mutations. All of these mutation types led to the same amino acid shift, indicating that the influence on sensitivity to QoIs was likely the same, regardless of the mutation type. It is possible that the population of TTA mutant type was selected first when QoIs were initially used and then accumulated subsequently.

Table 7. EC₅₀ values of *Alternaria solani* when subcultured on clarified V8 juice (CV8) agar media amended with and without 1 mg/liter of azoxystrobin (AZ) and 100 mg/liter of salicylhydroxamic acid (SHAM)

<i>A. solani</i>	Subculture transfer	Grown on CV8 with 1 mg/liter of AZ			Grown on CV8		
		Mean EC ₅₀ (mg/liter) ^x	Standard error ^y	<i>P</i> < 0.05 ^z	Mean EC ₅₀ (mg/liter)	Standard error	<i>P</i> < 0.05
Isolate As1	First	3.425	0.778	a	3.438	0.207	a
	Third	1.323	0.180	a	2.894	1.108	a
	Fifth	1.923	0.024	a	3.543	1.080	a
Isolate As2	First	0.247	0.035	b	0.037	0.017	c
	Third	0.277	0.076	ab	0.209	0.004	bc
	Fifth	0.452	0.024	a	0.034	0.017	c
Isolate As3	First	2.700	0.842	a	3.319	0.342	a
	Third	2.745	0.814	a	3.401	0.412	a
	Fifth	3.979	1.056	a	3.273	0.131	a

^x The EC₅₀ of each isolate was based on conidial germination rates on water agar amended with 0, 0.001, 0.01, 0.1, 1, and 10 ml/liter of AZ and 100 mg/liter of SHAM.

^y Standard error based on three replications.

^z Comparisons were made among the three subculture transfers on AZ and CV8 of each isolate. The same letter indicates no significant difference at the $\alpha = 0.05$ significance level based on the Tukey test.

Fig. 2. Comparison of *Alternaria alternata* mycelial growth at day 7 when transferred and grown on clarified V8 juice (CV8) amended with 1 mg/liter azoxystrobin (AZ) and 100 mg/liter of salicylhydroxamic acid (SHAM) (shown as black columns) and CV8 without AZ and SHAM (shown as gray columns) one, three, and five times. Different letters above the columns indicate significant differences at $\alpha = 0.05$ based on the Tukey test.

Table 8. EC₅₀ values of *Alternaria alternata* when subcultured on clarified V8 juice (CV8) agar media amended with and without 1 mg/liter of azoxystrobin (AZ) and 100 mg/liter of salicylhydroxamic acid (SHAM)

<i>A. alternata</i>	Subculture transfer	Grown on CV8 with 1 mg/liter AZ			Grown on CV8		
		Mean EC ₅₀ (mg/liter) ^w	Standard error ^x	<i>P</i> < 0.05 ^y	Mean EC ₅₀ (mg/liter)	Standard error	<i>P</i> < 0.05
Isolate Aa1	First	1.375	0.050	bc	0.479	0.015	c
	Third	3.206	1.421	b	0.487	0.082	c
	Fifth	8.521	0.804	a	0.662	0.049	c
Isolate Aa2	First	>10 ^z			>10		
	Third	>10			>10		
	Fifth	>10			>10		
Isolate Aa3	First	2.033	0.137	a	0.760	0.246	b
	Third	>10			0.535	0.110	b
	Fifth	>10			0.796	0.031	b

^w The EC₅₀ of each isolate was based on conidial germination rates on water agar amended with 0, 0.001, 0.01, 0.1, 1, and 10 ml/liter of AZ and 100 mg/liter of SHAM.

^x Standard error based on three replications.

^y Comparisons were made among the three subculture transfers on AZ and CV8 of each isolate. The same letter indicates no significant difference at the $\alpha = 0.05$ significance level based on the Tukey test.

^z EC₅₀ exceeded 10 mg/liter, so no statistical analysis could be conducted.

Overall, the frequency of *A. alternata* carrying G143A did not change throughout the 3 years of sampling, but the frequency varied between locations. A QoI application was considered the driving force of the higher frequency of QoI resistance in *A. alternata* isolates from the commercial potato fields (Samuel et al. 2011; Toffolatti et al. 2007). With QoI fungicide application, the overall frequency of *A. alternata* carrying G143A was significantly or moderately higher in the commercial fields where QoIs were applied than the organic field where QoIs were not applied over the years of this study. Without selection pressure, most of the *A. alternata* isolates carrying the G143A mutation might have originated from outside sources. For example, they may potentially be QoI resistant isolates windblown from another potato field or fields of other host crops where QoIs had been applied (McCartney et al. 1993; Rotem 1994).

The frequency of *A. alternata* isolates carrying the G143A mutation changed every year. The potential rate at which QoI resistance could develop in both *Alternaria* spp. is high. However, unlike the consistently high frequency of *A. solani* isolates carrying F129L, the frequency of *A. alternata* isolates carrying G143A mutation started low in the early season and increased in the later season. Because there was no fitness cost to carrying G143A (Fan et al. 2015; Karaoglanidis et al. 2011; Vega and Dewdney 2014), the sensitivity of *A. alternata* to QoI is unlikely to return. It is important to note that this trend is based on only two time points within the growing season and more sampling would be needed in order to better assess mutation frequencies. The increasing frequency of *A. alternata* isolates carrying G143A did not seem to be significantly correlated to the number of QoI applications made each year in the fields used in this study. This could be a result of the wide host range and conidial movement of *A. alternata* that may have complicated the process of selection of resistant isolates by QoI application (Tyman and Johnson 2014). Given that *A. alternata* has a broad host range, including Cucurbitaceae species, citrus, and *Syringa* species, to name a few (Mmbaga et al. 2011; Vakalounakis 1990; Vega et al. 2012), it is possible that there are wild-type populations maintained in alternative host species and that the conidia moved to the potato fields on prevailing wind currents after primary infection. Therefore, the frequency of *A. alternata* isolates carrying the mutation increased from early season to late season every year, but it never reached as high as that of *A. solani* isolates although they received the same fungicide application.

The high potential rate at which QoI resistance could develop for both *Alternaria* species was evidenced in the serial transfers of both species on AZ-amended media. When EC₅₀ was lower than the selection pressure, resistance could be selected for and accumulate quickly, as a significantly elevated EC₅₀ after one transfer on AZ-amended media was observed. Such phenomena were observed with *A. solani* isolate As2 and *A. alternata* isolates Aa1 and Aa3. The rapid selection was driven mainly by the abundant production of asexual conidia, as suggested by Fraaije et al. (2005). Resistance to AZ was shown by increasing mycelia growth from the first to the fifth transfers, but variations may occur and the EC₅₀ values of the subcultures in one transfer of one isolate may not be identical to another. The target of QoIs is cytochrome *b*, a mitochondrial gene. Mitochondria in cells divide unevenly and therefore the isolates transmit their resistant and sensitive types of cytochrome *b* genes to progeny in variable ratios (Van Leeuwen et al. 2008). The relative abundance of the wild-type allele of cytochrome *b* was found to influence the sensitivity level of the isolates to QoIs (Lesemann et al. 2006; Villani and Cox 2014), which may explain the difference in EC₅₀ when two isolates had the same codon types such as *A. alternata* isolates Aa2 and Aa3.

QoI resistance in both *Alternaria* species was stable for at least five generations, even when the selection pressure of QoI was removed. In the field, although QoI fungicides were not applied, (e.g., in 2015 in the Hancock field and 2016 in the Plover field), the frequency of *A. solani* isolates carrying the QoI resistance mutation was 100% and the frequency of *A. alternata* isolates carrying the QoI resistance mutation also showed an increasing trend from early season to late season. Such stability in QoI resistance was evidenced

in the serial transfers of QoI resistant isolates on AZ-amended media as well. When QoIs were not present, or the QoI concentration was lower than the EC₅₀ of the isolates, neither EC₅₀ values of these isolates nor the codon types of the 129 codon in their cytochrome *b* changed over five serial transfers. For example, the EC₅₀ of *A. solani* isolates As1 and As3 did not change over five serial transfers, and the EC₅₀ of all the isolates grown on CV8 did not change either. Similar phenomena were observed in other studies (Karaoglanidis et al. 2011; Pasche and Gudmestad 2008; Vega and Dewdney 2014), indicating that carrying the mutation conferring QoI resistance does not have a fitness cost for the pathogens. Therefore, mutations in cytochrome *b* conferring QoI resistance are stable in pathogen populations to some degree (Ishii et al. 2007; Lesemann et al. 2006; Samuel et al. 2011).

In summary, the results of this study showed that the frequency of *A. solani* isolates carrying QoI resistance mutations was consistently high in Wisconsin. The resistance in *A. alternata* isolates, however, showed a temporal pattern in which the resistance frequency started out low in early season and increased in late season. Because mutations that confer QoI resistance are associated with the mitochondrial cytochrome *b* gene, the relative abundance of mutations in the gene of one *Alternaria* isolate may be related to the level of its QoI resistance. The resistance to QoIs was stable in the *Alternaria* species for at least a few generations. Therefore, stopping QoI application for a few years may not help re-establish the sensitivity of the pathogens. QoIs may still be effective for controlling brown spot, but likely only early in the growing season (approximately June). Fungicides with alternative modes of action may be applied to achieve a more effective early blight and brown spot disease management.

Acknowledgments

We thank our grower collaborators for allowing us access to their fields for sampling and Dr. Monica Yu Chen for critically reviewing this manuscript.

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