Evaluation of QoI Fungicide Application Strategies for Managing Fungicide Resistance and Potato Early Blight Epidemics in Wisconsin

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ABSTRACT

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Potato early blight (*Alternaria solani*) is a yield-limiting disease and control depends primarily on multiple fungicide applications. Azoxystrobin, registered in the United States in 1999, initially provided outstanding early blight control. Within 3 years, approximately 80% of the total potato acreage was being treated with azoxystrobin and other quinone outside inhibitor (QoI), fungicides registered subsequently. *Alternaria solani* isolates with decreased in vitro sensitivity to azoxystrobin were detected in Wisconsin during 2001. Field experiments were conducted in 2001 to 2003 to evaluate season-long fungicide programs and test fungicide resistance management strategies. The fungicide program recommended to growers at that time, which consisted of three applications of azoxystrobin for weeks 1, 3, and 5 alternated with applications of chlorothalonil at label recommended rates, was effective in controlling early blight when conditions were conducive to disease development. Mean sensitivity in vitro of *A. solani* isolates from fungicide efficacy field experiments in 2001 to 2003 was numerically highest for isolates from the untreated control plots, chlorothalonil-alone plots, or plots treated with three applications of azoxystrobin alternated with chlorothalonil compared with other treatments tested. Three single-nucleotide polymorphisms (SNPs) can cause the F129L substitution (TTC to TTA, CTC, or TTG) that results in decreased sensitivity to azoxystrobin of *A. solani*. The TTA mutant was the most frequently recovered mutant type in the field experiments. The frequency of recovery of wild-type isolates in experiments was 22% in 2001, 4% in 2002, and 22% in 2003.

Additional keywords: quantitative PCR

Potato early blight, caused by the polycyclic Fungi Imperfecti *Alternaria solani* Sorauer, is an annual production concern for commercial potato growers in Wisconsin in years with intermittent wet and dry periods, especially in areas with overhead irrigation and frequent dew where disease pressure becomes particularly intense, resulting in reduced tuber yield reaching 20% (19). Disease severity can be especially severe in poorly nourished crops due to insufficient nitrogen fertilization (23). Early blight control depends primarily on multiple fungicide applications in the Midwestern United States, typically weekly applications on long-season potato cultivars (e.g., Russet Burbank). A reduction in pesticide inputs, coupled with the use of effective reduced-risk chemistries, is a desirable goal for commercial potato production, particularly in regions where environmental factors (e.g., temperature,

relative humidity, and so on) favor disease development and consequent calendar-based applications. The quinone outside inhibitor (QoI) fungicides (kresoxim-methyl, azoxystrobin, pyraclostrobin, trifloxystrobin, famoxadone, and fenamidone) offer a broad spectrum of activity, reduced environmental impact, and reduced toxicity to mammals compared with conventional materials used for the control of early blight on potato (e.g., chlorothalonil, mancozeb, and mefenoxam). The first QoI fungicides, kresoxim-methyl and azoxystrobin (AZ), were released commercially in Europe in the mid 1990s and the United States in the late 1990s (1,11,12). QoI fungicides disrupt mitochondrial respiration by binding to cytochrome *b*. An experimental use permit (EUP) and, subsequently, a Section 18 registration were granted by the Environmental Protection Agency (EPA) that allowed potato growers in Wisconsin to use AZ (Quadris; Syngenta Crop Protection, Greensboro, NC) for control of *A. solani* midseason in 1998, and AZ received full label registration for commercial use on potato in 1999. Due to the persistent use of AZ and other QoI fungicides alternated with chlorothalonil (CH) over the next six seasons after registration, the selection

pressure for the development of QoI fungicide resistance imposed on *A. solani* was relatively high on potato crops in Wisconsin and other regions of potato production in the United States (20). In Wisconsin during 2001 to 2003, approximately 80% of the total potato acreage was treated with QoI fungicides (average of three applications per year) alternated with CH or EBDC materials (e.g., maneb, mancozeb, and metiram).

Resistance to AZ first was described in isolates of *Blumeria graminis* f. sp. *tritici* in commercial wheat fields in Europe after only 2 years of product use (20), when disease control efficacy declined in many of these sites. No difference was detected in aggressiveness between AZ-resistant and -sensitive *B. graminis* isolates (5,25). Isolates resistant to QoI fungicides have been observed in other plant-pathogenic fungi at field and experimental sites after several years of intensive QoI use (2–5,10,14,17) (<http://www.frac.info/index>). Fungicide efficacy studies in Wisconsin over seven seasons have shown that AZ product efficacy for the control of potato early blight declined after commercial release (21).

The mode of resistance at the molecular and genetic levels and the existence of mutants resistant to the QoI fungicides have been known for several years in several organisms (4), including the yeast *Saccharomyces cerevisiae* (6,7). In *S. cerevisiae*, resistance to QoI fungicides was attributed to point mutations in two regions of the cytochrome *b* gene causing single amino acid changes in the Qo center coding region at amino acid positions from 127 to 147 and 275 to 296 (8,11). A similar single-point mutation was observed when DNA sequences of the cytochrome *b* were compared between QoI-sensitive and -resistant field isolates of several fungi (3,13,17). An amino acid substitution at position 129 from phenylalanine (F) to leucine (L) was observed in several plant-pathogenic fungi, including *A. solani*, and was associated with a decreased sensitivity to QoI compounds (3,13,17). Additionally, an amino acid substitution at position 143 from glycine (G) to alanine (A) rendered several species of plant-pathogenic fungi highly resistant to QoIs (2,3,10,15). The F129L substitution caused reduced sensitivity to QoIs whereas the G143A substitution resulted in a complete loss of sensitiv-

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ity or a lack of response ("resistant") (2,3,10,15). *A. solani* isolates with decreased in vitro sensitivity to AZ were detected in Wisconsin during 2001 from fungicide field experiments (21). During 2002 and 2003, a statewide monitoring of *A. solani* from commercial potato fields indicated an increase in frequency of recovery of isolates with reduced in vitro sensitivity to AZ and a wide distribution across the potato production regions of Wisconsin. This decrease in sensitivity was correlated with the presence of site-specific mutations at position 129 of the cytochrome *b* gene.

The effect of increased AZ applications coupled with practical fungicide resistance management tactics on experimental field populations of *A. solani* is not available in the literature. Disease pressure and the number of fungicide applications influence the evolution of fungicide resistance in many plant pathogens (5,22,25). There is no single resistance management strategy that is applicable to every disease situation. Therefore, the design, testing, and implementation of effective fungicide resistance management strategies are important components of current integrated pest management programs for the control of potato early blight in Wisconsin.

Due to stringent EPA regulations on fungicide label registration and fungicide resistance management concerns, fungicide label recommendations emphasizing resistance management strategies are implemented from the time of initial registration. Therefore, comprehensive field experiments to evaluate potential fungicide resistance management strategies for AZ were initiated. The objective of this study was to determine the impact of potential resistance management strategies consisting of alternations and tank mixtures of AZ with CH in experimental field experiments on (i) early blight disease progress, (ii) the in vitro sensitivity to AZ in *A. solani* isolates recovered from experimental field plots, and (iii) the frequency of wild-type and mutant isolates of *A. solani* recovered from experimental field experiments.

MATERIALS AND METHODS

Prior to use of AZ by commercial potato growers and these studies, potato leaves infected with *A. solani* were collected from commercial growers' fields representing major production regions throughout Wisconsin to determine baseline sensitivity to AZ. Experimental field experiments were established at the Hancock Agricultural Research Station (HARS) and treatments were designed to evaluate any differences in foliar early blight progression and area under the disease progress curves (AUDPC) in the field. Subsequently, sensitivity of *A. solani* isolates to AZ was determined using an in vitro assay based on relative germination of conidia on fungicide-amended agar. Quantitative poly-

merase chain reaction (Q-PCR) assays were employed to evaluate whether reduced sensitivity to AZ was correlated with site-specific mutations in the cytochrome *b* gene, caused by single-nucleotide polymorphisms (SNPs).

Materials for in vitro sensitivity to AZ. Technical-grade AZ (95.3% a.i.) was obtained from Syngenta Crop Protection. Bacto Agar was from Difco Laboratories (Detroit). All other chemicals were from Sigma-Aldrich (St. Louis) unless otherwise noted.

Field experiments. Annual field experiments were established to evaluate the effects of alternations and tank mixtures of AZ (Q = Quadris 2.08 F; Syngenta Crop Protection; AZ, 0.46 liters/ha) with CH (BZ = Bravo Zn 4.17 F; Syngenta Crop Protection; CH + zinc, 2.5 liters/ha) and Bravo WS (BW; Syngenta Crop Protection; CH + weather stick, 1.8 liters/ha) for early blight control. Plots were planted between 23 and 29 April from 2001 to 2003 at the HARS in central Wisconsin. Fungicide treatments consisted of 0 to 6 applications of AZ out of a total of 10 applications during the growing season (Table 1). Certified Russet Burbank US#1 seed tubers (Wisconsin Seed Potato Certification) were mechanically cut into approximately 62-g seed pieces and allowed to heal prior to planting. A randomized complete block design with four replications was used for the experiments, with each plot consisting of four 7-m-long rows spaced 1 m apart, with tubers placed 38 cm apart in the row. Drive rows for pesticide application were placed in adjacent plots within field experiments to avoid soil compaction and provide a source for inoculum for dispersal of *A. solani*. Fungicide treatments were applied to all four rows of each plot at weekly intervals starting between 19 and 26 June and ending between 20 August and 4 September, depending on planting dates, for a total of 10 weeks (Table 1). Treatments were applied in a volume equivalent to 327 liters of water/ha at 276 kPa, using Tee Jet hollow disc cone D3-23 nozzles (15 nozzles at 20-cm spacing) with a tractor-mounted boom sprayer pressurized with an air compressor. Plots were not inoculated but relied on natural sources of *A. solani* for disease establishment. Disease severity was assessed weekly on four arbitrarily selected plants per row from 19 June through 4 September using the Horsfall-Barratt rating scale of 0 (no infection) to 11 (all foliage and stems dead) and converted to percent. Rainfall was measured during the growing season and an additional 30 to 70 cm of water was applied as needed via overhead sprinkler irrigation in 24 to 40 applications over the 3 years (27 May to 13 September), 2001 to 2003.

Sampling procedure. AZ first was used in commercial fields in Wisconsin during 1998 in a tightly controlled EUP program.

A. solani collected from fields prior to the application of AZ constituted the baseline isolates and were screened in a previous study (22). The in vitro fungicide sensitivity of fungal isolates was investigated in the laboratory. During 2001, 2002, and 2003, 20 potato leaves infected with *A. solani* were collected randomly in a standard W pattern from fungicide trial plots at HARS near the end of the growing season. Treatment plots (four replicates) received up to six applications per season of Quadris in 2001 and 2002, and up to three applications in 2003 (Table 1). Treatments, as described previously, were tested to identify potential differences in foliar early blight progression and AUDPC in the field. Mean effective concentration of AZ (mg/liter) needed for 50% inhibition of spore germination (EC_{50}) of *A. solani* collected in the treated plots (four replicates) then was determined in the laboratory using individual monoconidial isolates of *A. solani* (394 isolates total).

From each leaf, one lesion was cut with a cork borer; the lesions were chosen visually from similar sizes and in similar stages of sporulation. All leaf disks derived from a particular fungicide treatment plot were placed onto water agar media (WA) amended with streptomycin for sporulation induction and positive identification (24). One conidium of *A. solani* from each sporulating lesion was transferred to clarified V8 (CV8) amended with $CaCO_3$ agar (100 ml of CV8, 900 ml of distilled H_2O , 15 g of Bacto Agar, and 1.5 g of $CaCO_3$) in 100-mm petri plates, placed under fluorescent light with a 24-h photoperiod at 20°C for 10 to 14 days, and subsequently stored at -20°C on colonized filter paper to reduce effects of subculturing and for further analyses.

In vitro assay of isolate sensitivity to AZ fungicide. An in vitro assay was developed based on relative conidial germination on AZ-amended media. Sensitivity of germinating conidia to AZ, derived from monoconidial isolates, was determined for isolates collected from the baseline sample and experimental field trial locations described above. Sensitivity of germinating conidia was assessed on WA (1,000 ml of distilled H_2O and 10 g of Bacto Agar) amended with AZ. AZ (a.i.) was dissolved in acetone and added to the WA at concentrations of 0.00, 0.001, 0.01, 0.1, 1, and 10 mg/liter. Additionally, salicylhydroxamic acid (SHAM) dissolved in methanol was added to the WA at 100 mg/liter. SHAM inhibits the alternative oxidase (AOX) pathway that has been suggested as a possible mode of QoI resistance in vitro in other fungi (31). Control medium included equal concentrations of acetone and methanol as fungicide-amended WA.

A hemacytometer was used to adjust the conidial suspension to 1×10^4 conidia per ml, and a 50- μ l aliquant was spread on the surface of AZ alone or AZ + SHAM-

amended media. Germination of 50 conidia was assessed at each concentration (two replications) after incubation under continuous light for 4 h at 26°C. A conidium was rated as germinated if a normally developing germ tube was at least equal to the total length of a conidium (beak and tail), if an appressorium formed at the tip of the germ tube, or if multiple germ tubes developed. Isolate sensitivities were expressed as relative germination (RG) (16), which is defined as the ratio of conidia germinating in the presence of AZ to those germinating in the absence of AZ $\times 100\%$. Isolate sensitivity expressed in mg/liter was determined by the EC_{50} . EC_{50} values were determined by interpolation of the 50% intercept, based on regression of the arcsine of RG on the $\log_{10}$ -transformed fungicide concentration.

SNP detection assay. A SNP detection assay using a Q-PCR procedure was employed to evaluate whether shifts in sensi-

tivity of *A. solani* isolates to AZ were correlated with an SNP in the cytochrome *b* gene. Genomic DNA extractions from plant (herbarium specimens) and fungal material (10- to 12-day-old cultures) was prepared using either DNeasy Plant Minikit (Qiagen, Valencia, CA) or Bio101

Fast DNA Kit (Qbiogene, Carlsbad, CA) according to the manufacturers' protocols. A subsample of *A. solani* isolates (160 isolates total) collected from fungicide field experiments was used in the SNP detection assay. Genomic DNA, conidia, or mycelia were stored on dry ice and sent

Table 2. Primers and fluorescent-labeled probes used in a real-time polymerase chain reaction assay to detect single-nucleotide polymorphisms in the cytochrome *b* gene of *Alternaria solani*^z

Primers, probes	Sequence (5' to 3')
129 Forward primer	GAACATTTGGTACTGTTATCTTTATCTTAATGATG
129 Reverse primer	AAGACATTTGCCATAAGGAAGAA
143 Forward primer	GTATGTTCTTCCTTATGGGCAAATG
143 Reverse primer	CTCACTTTGTTTATGTTATTTAAACCAAGAATG
129 WT probe	[FAM] TACAGCTTTCCTGGGTTA
129 MUT1 probe	[VIC] TACAGCTCTCCTGGGTTT
129 MUT2 probe	[VIC] TACAGCTTACTGGGTTAT
129 MUT3 probe	[VIC] TACAGCTTTGCTGGGTTT
143 WT probe	[FAM] TCTTTATGAGGTAATTT
143 MUT probe	[VIC] TCTTTATGAAGCTAAATTT

^z Sequences from Syngenta International Patent WO02081742.

Table 1. Collection year, applied fungicides, application history, area under the disease progress curve (AUDPC), mean effective concentration of azoxystrobin (AZ) needed for 50% inhibition of spore germination (EC_{50}), and mutations in the cytochrome *b* gene in isolates of *Alternaria solani* recovered from fungicide field experiments at Hancock Agricultural Research Station during 2001, 2002, and 2003

Year, treatment ^y	Quadris (weeks) ^w	QoI ^x	AUDPC ^y	No. of isolates	Mean EC ₅₀ (mg/liter) ^z	Wild type ^t	Mutant type ^u		
						TTC	TTA	CTC	TTG
2001									
Untreated	N/A	0	0.243 a	20	0.27 c	4	3	0	1
BZ	N/A	0	0.149 b	20	0.22 c	9	1	0	0
Q/BZ	1	1	0.05 c	20	0.47 bc	1	5	1	0
Q/BZ	1 & 3	2	0.084 c	20	1.01 bc	1	6	1	0
Q/BZ	1,3 & 5	3	0.047 c	20	0.47 bc	0	7	0	0
Q/BZ	1,3,5 & 7	4	0.055 c	20	0.53 bc	0	4	2	1
Q/BZ	1,3,5,7 & 8	5	0.049 c	20	2.39 a	0	7	2	0
Q/BZ	1,3,5,7-9	6	0.059 c	20	1.26 b	0	9	2	0
<i>P</i> value	...	...	<0.0001	...	<0.0001	...	...	...	...
LSD	...	...	0.0397	...	1.976	...	...	...	...
2002									
Untreated	N/A	0	0.07	20	0.54 bc	4	1	0	1
BZ	N/A	0	0.038	20	0.48 bcd	0	3	0	4
Q/BZ	1	1	0.039	20	0.83 a	0	6	1	1
Q/BZ	1 & 3	2	0.029	20	0.72 ab	0	4	0	4
Q/BZ	1,3 & 5	3	0.056	20	0.43 cd	0	8	0	2
Q/BZ	1,3,5 & 7	4	0.041	20	0.56 bc	0	6	1	3
Q/BZ	1,3,5,7 & 8	5	0.032	20	0.64 abc	0	4	2	2
Q/BZ	1,3,5,7-9	6	0.038	20	0.66 abc	0	7	0	1
Q+BW/BW	1,3 & 5	3	0.026	10	0.28 d	0	6	0	3
<i>P</i> value	...	...	0.1313	...	0.0002	...	...	...	...
LSD	...	...	N/A	...	1.975	...	...	...	...
2003									
Untreated	N/A	0	0.292 a	11	0.53	0	2	1	0
BZ	N/A	0	0.208 bc	6	0.54	1	1	1	0
Q/BZ	1	1	0.265 ab	11	0.81	1	3	0	0
Q/BZ	1 & 3	2	0.223 bc	11	0.9	0	1	0	3
Q/BZ	1,3 & 5	3	0.217 bc	13	0.5	0	0	0	2
Q+BW/BW	1,3 & 5	3	0.17 c	12	0.78	2	1	0	0
<i>P</i> value	...	...	0.0135	...	0.094	...	...	...	...
LSD	...	...	0.0651	...	N/A	...	...	...	...

^t A subsample of isolates was screened in the single-nucleotide polymorphism detection assay. Wild-type isolates are considered sensitive to AZ and constituted the 1998 baseline for this study. Sequencing of the cytochrome *b* gene revealed a phenylalanine at amino acid position 129 of the transcribed protein.

^u Mutant-type isolates have a decreased sensitive to AZ and the frequency of their recovery increased after commercial release of quinone outside inhibitor (QoI) fungicides. Sequencing of the cytochrome *b* gene revealed a leucine at amino acid position 129 of the transcribed protein.

^v Treatment chemicals include Q = Quadris 2.08 F (AZ, 0.46 liters/ha), BZ = Bravo Zn 4.17 F (chlorothalonil + zinc, 2.5 liters/ha), and BW = Bravo WS (chlorothalonil + weather stick, 1.8 liters/ha).

^w Weeks that Quadris was applied. In the remaining weeks, Bravo Zn or Bravo WS was applied; N/A = not applicable.

^x Number of QoI applications per season.

^y Relative AUDPC based on the assessments of disease severity. Values were divided by the number of days in the season and expressed as a proportion of the maximum observed AUDPC. Values followed by the same letter are significantly different, based on Fisher's protected least significant difference (LSD) test; N/A = not applicable.

^z Values followed by the same letter are significantly different, based on Fisher's protected LSD test; N/A = not applicable.

to Syngenta, Vero Beach, FL or Jealott's Hill, UK for SNP detection assays.

One SNP can cause the G143A substitution (GCT to GGT) whereas three different SNPs can cause the F129L substitution (TTC to TTA, CTC, and TTG). Primers and fluorescent-labeled minor-groove-binding probes, to be used in a real-time PCR assay (Table 2), were designed for the detection of the four SNPs responsible for the G143A and F129L amino acid substitutions in *A. solani* (International Patent Publication No. WO02081742). Each hybridization reaction consisted of 5 µl of template DNA, 2× TaqMan mastermix (Applied Biosystems Inc., Foster City, CA), 18 µM forward and reverse primer, and 2 µM wild-type and mutant probe in a 25-µl reaction volume. An ABI prism 7700 real-time PCR instrument (Applied Biosystems Inc.) was used in the SNP detection assays on site at Syngenta, Vero Beach, FL. Amplifications were run with the following thermal-cycler parameters: 50°C for 2 min, 95°C for 10 min, 35 cycles of 92°C for 15 s, and 60°C for 1 min. Data were collected during the 60°C step with 5-carboxy-X-rhodamine (ROX) as the reference standard.

Data analysis. Disease severity ratings were converted to percentages using mid-points of Horsfall-Barratt class intervals. Data for each observation date was plotted on a graph (disease severity versus time) and AUDPC was calculated for each treatment providing a measure of the relative severity of disease throughout the season. A disease rating of 100% severity for the entire season would produce a maximum possible AUDPC value of $100\% \times N$, where N = the number of days in the season. Dividing the actual AUDPC values by this maximum possible value would result in the relative AUDPC; on a scale of 0 to 1.0, a disease rating of 100% severity for AUDPC value of 1.0 represents the maximum possible relative AUDPC. All relative AUDPC values are expressed as a proportion of this value and were compared using one-way analysis of variance (ANOVA), and Fisher's protected least significant difference (LSD) procedure was used for mean separation.

$\log_{10}$ -transformed mean EC_{50} values were compared using one-way ANOVA and Fisher's protected LSD procedure for mean separation. Frequencies of recovery of each of the mutant types were com-

pared, using a χ^2 test, to assess for potential differences among treatments. All statistical analyses were performed with Minitab (v.10.5 Xtra Power; Minitab Inc., State College, PA) or SAS (v.8.0; SAS Institute Inc., Cary, NC). Real-time PCR assay results were analyzed using SDS software (v.1.7; Applied Biosystems Inc.).

RESULTS

Field experiments. Final disease severity in untreated plots was 88.7, 81.3, and 84.6% during 2001, 2002, and 2003, respectively. During 2001, AUDPC values from treatments with one to six applications of AZ were significantly lower than the untreated control and CH alone, but were not significantly different from one another (Table 1). During 2002, there were no significant differences in AUDPC values among treatments (Table 1). Fungicide treatments with multiple applications of AZ did not provide additional disease control; therefore, treatments with more than three AZ applications were eliminated in 2003. During 2003, AUDPC values from all fungicide treatments, except for the treatment with one application of AZ, were significantly lower than the untreated control. Furthermore, the AUDPC for all tested alternations, including the tank mix of AZ and CH, were not significantly different from those plots treated with CH alone (Table 1).

In vitro assay of isolate sensitivity to AZ fungicide. EC_{50} values representing in vitro sensitivity of baseline *A. solani* isolates to AZ ranged from 0.012 to 0.997 mg/liter to AZ without SHAM. Sensitivity of baseline isolates in the presence of SHAM ranged from 0.008 to 0.069 mg/liter for AZ. A wide range of EC_{50} values was observed for *A. solani*, with a mean sensitivity of 0.179 mg/liter (standard deviation [SD] = 0.088) for AZ alone. Mean sensitivity (EC_{50}) of baseline isolates in the presence of AZ and SHAM was significantly lower, 0.031 mg/liter (SD = 0.1) and was less variable than for AZ alone.

EC_{50} values of isolates collected from fungicide field experiments in 2001, 2002, and 2003 were significantly higher than those of the baseline isolates ($P < 0.0001$, data not shown). Mean EC_{50} values for isolates collected in 2001 from plots treated with one to four applications of AZ were not significantly different from those of isolates from the untreated control plots and plots treated with CH alone (Fig. 1; Table 1). Mean EC_{50} values from treatments with five or six applications of AZ were significantly higher compared with the untreated control plots or from plots treated with CH alone, but there was no difference in mean EC_{50} among isolates from plots treated with one, two, three, four, and six applications of AZ (Fig. 1; Table 1).

Mean EC_{50} values of *A. solani* isolates collected in 2002 from plots treated with

Fig. 1. Frequency distributions of in vitro sensitivity to azoxystrobin (AZ) of *Alternaria solani* isolates collected in 2001 from potato leaves exposed to different fungicide treatments in the field. Sensitivity expressed as the $\log_{10}$ -transformed concentration of AZ needed for 50% inhibition of spore germination (EC_{50} , mg/liter) based on an in vitro conidia germination assay on medium amended with AZ and salicylhydroxamic acid. Q = Quadris (AZ) and BZ = Bravo Zn (chlorothalonil + zinc).

two to six applications of AZ were not significantly different from the untreated control and CH alone (Fig. 1; Table 2). Mean EC_{50} values from plots treated with one application of AZ and tank mixtures of AZ or CH were significantly different from each other, and the latter was lower than the mean EC_{50} of isolates from the untreated control and from those treated with CH alone (Fig. 2; Table 1). Mean sensitivity in vitro of isolates from plots treated with three applications of AZ was significantly different from treatments consisting of one, two, four, five, and six applications of AZ, but was not different from mean EC_{50} of isolates from plots treated with three tank-mixed applications of AZ during 2002 (Fig. 2; Table 1). Several treatments that included three applications of AZ in prepack and tank mixtures at various rates alternated with CH were tested only in 2002 (*data not shown*). The application of a prepack mixture of AZ and CH at a low rate (1.4 liters/ha) resulted in a wider range of sensitivities to AZ and a significantly lower mean EC_{50} than all other tested mixtures in 2002, but the mean sensitivity was not significantly different from those isolates from plots treated with one to six applications of AZ ($P = 0.05$, *data not shown*). Fungicide field experiments in 2003 revealed no statistical difference in mean EC_{50} values among treatments (Fig. 3; Table 1).

SNP detection assay. All of the isolates collected in 1998 were wild type at amino acid positions 129 (F; codon TTC) and 143 (G; codon GGT). All of the isolates collected throughout the course of this study were wild type at position 143, whereas all

three possible SNP combinations (TTA, CTC, and TTG) were detected at position 129 from the recovered *A. solani* isolates from field experiments in 2001, 2002, and 2003 (Table 1). The majority of the 2001 (77%), 2002 (95%), and 2003 (79%) isolates carried an SNP mutation. The most common mutant type recovered in 2001 was TTA followed by CTC and TTG (Ta-

ble 1). The most common mutant type recovered in 2002 and 2003 was TTA followed by TTG and CTC (Table 1).

The results from the molecular assays indicated that there were significant differences in the frequency of mutant types detected between isolates from the untreated control and plots treated with three to six applications of AZ, and between all

Fig. 3. Frequency distributions of in vitro sensitivity to azoxystrobin (AZ) of *Alternaria solani* isolates collected in 2002 from potato leaves exposed to different fungicide treatments in the field. Sensitivity expressed as the $\log_{10}$ -transformed concentration of AZ needed for 50% inhibition of spore germination (EC_{50} , mg/liter) based on an in vitro conidia germination assay on medium amended with AZ and salicylhydroxamic acid. Q = Quadris (AZ) and BZ = Bravo Zn (chlorothalonil + zinc) BW = Bravo WS (chlorothalonil).

Fig. 2. Frequency distributions of in vitro sensitivity to azoxystrobin (AZ) of *Alternaria solani* isolates collected in 2002 from potato leaves exposed to different fungicide treatments in the field. Sensitivity expressed as the $\log_{10}$ -transformed concentration of AZ needed for 50% inhibition of spore germination (EC_{50} , mg/liter) based on an in vitro conidia germination assay on medium amended with AZ and salicylhydroxamic acid. Q = Quadris (AZ) and BZ = Bravo Zn (chlorothalonil + zinc) BW = Bravo WS (chlorothalonil).

tested alternations and CH alone in 2001 (Tables 1 and 3). There was no difference in the frequency of recovered mutant types detected among tested alternations of one to six applications of AZ in 2001. In 2002, frequencies of mutant types recovered from all tested fungicide treatments were significantly different from the untreated control (Tables 1 and 3). Conversely, there were no significantly significant differences in frequency of mutants among all fungicide treatments (Tables 1 and 3). In 2003, there was no difference in the frequency of recovered mutant types detected among all tested fungicide treatments (Table 1).

DISCUSSION

This project was designed to develop information needed to augment current disease and crop management systems, with a focus on testing existing resistance management strategies for the control of potato early blight in Wisconsin. Results based on data collected during 2001 indicated that one to three applications of AZ alternated with CH in a 9-week application program gave the best early blight control. Moreover, growers have observed a boost in tuber yield when three applications of AZ were included in their management program (W. R. Stevenson, *unpublished*). During 2002, there was greater variability in disease severity among replications and less disease, possibly due to environmental conditions unfavorable for disease occurrence and pathogen spread. During 2003,

AUDPC values for all treatments were numerically similar to the untreated control in contrast to 2001 and 2002, which may be attributed to a higher frequency of isolates with decreased sensitivity to AZ, variability in disease severity, and environmental conditions favorable for early blight disease epidemics.

This study substantiates the results of a statewide monitoring of populations of *A. solani* in Wisconsin, which confirmed that a directional selection favoring a single allele and a population of isolates with a reduced response to AZ has occurred since EPA label registration in 1998 (baseline year). This selection in *A. solani* seems to occur at the population level, leading to a significant change in sensitivity of the population of *A. solani* relative to the baseline. Furthermore, the data from the present study shows a lack of significant differences in sensitivity to AZ among mutant types. The distribution of EC₅₀ values of the isolates used in this study may resemble a quantitative sensitivity shift. Unlike the case of DMIs (9), available evidence does not support a hypothesis of multigenic resistance to QoIs in *A. solani* (5,18,25). Additionally, AZ targets mitochondria that may vary in number among conidial cells, and the frequency of mutant mitochondria within cells also may be expected to differ.

Comprehensive field and laboratory experiments conducted over the course of three potato growing seasons revealed that the reduction in sensitivity was consis-

tently associated with site-specific amino acid substitutions in the cytochrome *b* gene. These were caused by three different SNPs at position 129 in cytochrome *b*, where phenylalanine is substituted with leucine. This study, in contrast to other studies (5,18,25), indicates differences in codon substitutions responsible for the F129L mutation in *A. solani*. The results of this research and the statewide monitoring study during 2001 to 2003 (22) indicate that selection has occurred for a population of isolates with mean in vitro sensitivities to AZ (EC₅₀s) 20-fold higher than baseline isolates of *A. solani* collected in 1998. Mean sensitivity of *A. solani* isolates was not different between 2001, 2002, and 2003. During 2002 and 2003, there was no correlation between AUDPC values and mean sensitivity to AZ. Compared with 2001 and 2003, greater variations in EC₅₀ values were observed among all fungicide treatments in 2002 (Table 1). This variation possibly is due to a variation in the recovery of wild-type *A. solani* isolates in plots treated with fungicides coupled with lower early blight disease severity and AUDPC values. Additionally a lack of statistical differences among all treatments was observed in 2002 due to high variability among replications. Although this trend was not consistent among years, no wild-type *A. solani* isolates were recovered in 2002 from experimental plots treated with AZ fungicide. Conversely, in 2001 and 2003, wild-type isolates were recovered from some but not all fungicide-treated plots. These results further reinforce the concept of a selective disadvantage for wild-type isolates of *A. solani* imposed by repeated fungicide applications regardless of how conducive the environmental conditions are for early blight.

Q-PCR assays performed on the 1998 baseline *A. solani* isolates indicated that all baseline isolates exhibited the wild-type genotype. None of the isolates collected in 1998, 2001, 2002, or 2003 exhibited mutations at position 143 in the cytochrome *b* gene, including isolates with elevated in vitro EC₅₀ values. In 2002, the frequency of wild types recovered from the Wisconsin statewide survey and experimental field populations was the same (4% of isolates). In contrast, no wild-type isolates were recovered in 2003 from commercial fields, but were present in our field experiments. Over the past five growing seasons (2000 to 2004), the efficacy of AZ has declined in fungicide evaluations (26–30), suggesting that a directional selection favoring a phenotype with a reduced response to AZ has occurred in populations of *A. solani* in Wisconsin subsequent to commercial release of Quadris. The fact that wild-type isolates of *A. solani* were recovered at a higher frequency from field research experiments than commercial fields suggests that the untreated guard rows in our fungicide trial plots provided a refuge for iso-

Table 3. Comparisons of frequency of wild-type and mutant isolates characterized by the amino acid substitution F129L in isolates of *Alternaria solani* recovered from potato leaves exposed to different fungicide treatments in field experiments in 2001 and 2002^y

Treatment ^z	1	2	3	4	5	6	7	8	9
2001									
1	...	–	–	–	+	+	+	+	...
2	...	...	+	+	+	+	+	+	...
3	...	...	...	–	–	–	–	–	...
4	...	...	...	...	–	–	–	–	...
5	...	...	...	...	...	–	–	–	...
6	...	...	...	...	...	...	–	–	...
7	...	...	...	...	...	...	...	–	...
8	...	...	...	...	...	...	...	...	...
2002									
1	...	+	+	+	+	+	+	+	+
2	...	...	–	–	–	–	–	–	–
3	...	...	...	–	–	–	–	–	–
4	...	...	...	...	–	–	–	–	–
5	...	...	...	...	–	–	–	–	–
6	...	...	...	...	...	–	–	–	–
7	...	...	...	...	...	...	–	–	–
8	...	...	...	...	...	...	...	–	–
9	...	...	...	...	...	...	...	...	–

^y Symbols: + indicates a significant difference between treatments using χ^2 analysis ($\alpha = 0.05$) and – indicates no difference. No significant differences in frequency of recovered isolates among treatments were detected in 2003.

^z Treatment chemicals include Q = Quadris 2.08 F (azoxystrobin, 0.46 liters/ha), BZ = Bravo Zn 4.17 F (chlorothalonil + zinc, 2.5 liters/ha), and BW = Bravo WS (chlorothalonil + weather stick, 1.8 liters/ha). Treatment 1 = untreated; treatment 2 = BZ; treatment 3 = Q, week 1/BZ remaining; treatment 4 = Q, weeks 1 and 3/BZ remaining; treatment 5 = Q, weeks 1, 3, and 5/BZ remaining; treatment 6 = Q, weeks 1, 3, 5, and 7/BZ remaining; treatment 7 = Q, weeks 1, 3, 5, 7, and 9/BZ remaining; treatment 8 = Q, weeks 1, 3, 5, 7–9/BZ remaining; treatment 9 = Q + BW, weeks 1, 3, and 5/BW remaining.

lates sensitive to AZ. There was a statistical difference in the frequency of mutant types (TTA, CTC, and TTG) detected among selected treatments in 2001 and 2002 as well as between years at field trial sites. These results suggest that, as the selection pressure is maintained on *A. solani* isolates with a reduced sensitivity to AZ, the frequency of TTG mutants may increase over CTC mutants; however, it remains unclear whether this is due to internal or external factors such as the cellular increase in mutant mitochondria or an influx from nearby grower's fields. However, results from statewide monitoring indicated that mean in vitro sensitivities to AZ (EC₅₀ values) among different mutant types were not significantly different from one another.

Results from the Q-PCR test were further confirmed by results of in vitro sensitivity tests demonstrating that EC₅₀ values were lowest in the untreated control, CH alone, and three applications of AZ. This was due in large part to less variability in the range of EC₅₀ values and a high frequency of recovery of isolates with sensitivity similar to baseline isolates collected prior to commercial use of QoIs. Effective disease control and a lower variability in EC₅₀ values usually was achieved in all three growing seasons in field plots treated with AZ and CH alternations that strictly adhered to the current label requirements for the use of QoI fungicides on potato. These requirements include: follow resistant management guidelines and do not apply more than one application of Quadris or other group 11 (QoI) fungicides before alternation with a fungicide that is not in group 11. In light of the results from this research, commercial potato growers in Wisconsin have continued to integrate these concepts in resistance management strategies.

Results from this study and statewide monitoring (22) further confirmed that a directional selection favoring isolates of *A. solani* with a reduced response to AZ is associated with the F129L substitution. An amino acid substitution at position 129 from phenylalanine (F) to leucine (L) previously was shown to be responsible for a decrease in sensitivity to AZ in several fungal species, including *A. solani* (5,18,25). Another amino acid substitution at position 143 from glycine (G) to alanine (A) rendered isolates of several fungal species resistant to QoIs (2,3,10,15). The F129L substitution generally causes a low magnitude decrease in sensitivity to QoIs or a reduced response ("intermediate resistance"), whereas the G143A substitution results in a high-magnitude decrease in sensitivity or no response ("resistant"). The reduced sensitivity responses in the sensitivity assay were associated consistently with molecular changes in the pathogen genome and vice versa. Thus, molecular probes may provide a reliable

tool to quickly detect QoI resistance or reduced sensitivity in field isolates of *A. solani* in future studies and in the further design, testing, and implementation of effective and durable fungicide-resistant management strategies for the control of early blight.

It appears that the risk for evolution of resistance to QoI fungicides remains high in plant-pathogen populations, including foliar diseases (e.g., powdery mildews, downy mildews, and rusts), for which numerous and frequent application of fungicides is the primary means of control. Potato early blight likely will continue to be an annual production concern and, because control of this polycyclic disease depends on multiple fungicide applications, the selection pressure on *A. solani* for fungicide resistance likely will remain high not only in Wisconsin but throughout the United States. Practical fungicide resistance management strategies are important for the continued viability of AZ and other QoI fungicides used for the control of early blight. This project used a combination of fungicide efficacy field experiments, laboratory (in vitro) sensitivity assays, and molecular (Q-PCR) research to study the effects of fungicide resistance management tactics as an added component of control measures for early blight of potato. The study found that, in our experimental field evaluations, there remains a high frequency of *A. solani* isolates with reduced sensitivity to AZ. Further confirmation of the mechanism involved in reduced sensitivity to strobilurin fungicides in *A. solani* and the selection pressure imposed by increased applications of QoIs for the control of early blight provides a valuable baseline of information. Moreover, this study found no added benefit in disease control with more than three applications of AZ. This is the first study to test the impact of increased number of QoI applications on *A. solani* in controlled field experiments. Reducing the important economic risk due to decreased product efficacy that results from the evolution of fungicide resistance and subsequent unexpected control failures is useful for the commercial production of potato.

The data from this study highlight the impacts of selection for populations of *A. solani* isolates with a reduced response to AZ in Wisconsin relative to the 1998 baseline. This is similar to findings conducted in other potato production areas with a history of AZ use (18). Results from both the Q-PCR and the in vitro sensitivity assay are highly correlative, indicating that reduced sensitivity of *A. solani* to AZ is induced by a point mutation at position 129 of the cytochrome *b* gene resulting in a change of the amino acid phenylalanine (F) to leucine (L). Further studies on the population biology of *A. solani* may be valuable in developing use recommendations for QoI fungicides in areas where

isolates with decreased sensitivity have been detected. Additionally, studies on the possible selective disadvantage associated with wild-type isolates of *A. solani* in years when environmental conditions are not favorable for disease and pathogen dispersal deserve future consideration. This study is further confirmation that a reduction in the total number of AZ applications may result in a reduction of selection pressure for *A. solani* mutants with decreased sensitivity to AZ. Effective disease control typically was achieved when growers strictly adhered to the current Quadris label recommendations for the use of QoI fungicides. Therefore, if growers wish to continue using AZ and other QoI fungicides, it is recommended to (i) limit the number of QoI applications to one-third of the total number of sprays during a season; (ii) apply QoI fungicides early in the growing season alternated (no consecutive applications), tank mixed, or, when available, as prepack mixes with compounds of different chemistries; and (iii) never use QoI fungicides in a curative manner.

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