Quantifying the Effects of a G137S Substitution in the Cytochrome *bc₁* of *Venturia effusa* on Azoxystrobin Sensitivity Using a Detached Leaf Assay

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Abstract

The quinone outside inhibitor (QoI) fungicides are known for their inherently high resistance risk owing to substitutions in amino acid residues 129, 137, or 143 of the cytochrome *b* gene of phytopathogens. In *Venturia effusa*, cause of pecan scab, an intron adjacent to position 143 likely reduces this risk; however, the effects of a recently discovered substitution at position 137 (G137S) are unknown. Traditional in vitro assays are not useful for determining sensitivity of isolates of *V. effusa* to the QoI fungicides, owing to the fungitoxic effects of required alternative oxidase inhibitors. A detached leaf assay was developed to quantify the sensitivity of 59 isolates to azoxystrobin: 45 wild-type isolates and 14 carrying

G137S. Isolate EC₅₀ values ranged from <0.0001 to 0.3047 µg/ml; EC₅₀ values for wild-type isolates ranged from <0.0001 to 0.2007 µg/ml (median 0.0023 µg/ml), whereas EC₅₀ values for G137S isolates ranged from 0.0033 to 0.3047 µg/ml (median 0.0178 µg/ml). The median EC₅₀ value for G137S isolates was significantly greater than that of the wild-type isolates; however, there was overlap between the two groups. This is the first report of sensitivity of *V. effusa* isolates to a QoI fungicide and evidence of G137S as a potential mechanism of partial resistance. However, although a complete control failure is unlikely, the impact of this substitution on QoI efficacy in Georgia pecan orchards remains to be determined.

Management of pecan scab, caused by *Venturia effusa* (G. Winter) Rossman & W.C. Allen (syn. *Fusicladium effusum*), relies on the application of preventive fungicides (Brock and Bertrand 2007a). This practice typically consists of 10 or more applications made every 10 to 14 days beginning at bud break and continuing until pollination, and then every 14 to 21 days until shell hardening occurs (Brock and Bertrand 2007b). During periods of frequent rainfall, fungicides are often applied as frequently as every 7 to 10 days, thereby increasing the total number of applications during the growing season (Brock et al. 2007; Latham 1995). The quinone outside inhibitor (QoI; Fungicide Resistance Action Committee [FRAC] code 11) fungicides have played a pivotal role in pecan scab management programs since the late 1990s as both stand-alone products and in formulated mixtures, often in combination with a demethylation inhibitor fungicide (DMI; FRAC code 3). There are a number of commercially available stand-alone QoI fungicides labeled for use on pecan in the United States that contain azoxystrobin (e.g., Abound 2.08F; Syngenta Crop Protection, Greensboro, NC), kresoxim-methyl (e.g., Sovran; BASF Corporation, Research Triangle Park, NC), or pyraclostrobin (e.g., Headline; BASF Corporation) (Brock et al. 2007).

The QoIs are mitochondrial respiration inhibitors that target the cytochrome *bc₁* enzyme complex (Fernández-Ortuño et al. 2008). By binding to the quinol oxidation site of the enzyme complex, the fungicide effectively blocks electron transport, which halts adenosine triphosphate production and results in an energy deficit in fungal cells (Bartlett et al. 2002; Fernández-Ortuño et al. 2008; Gisi et al. 2002). The QoIs are considered to have an inherently high risk of resistance owing to their site-specific mode of action and history of resistance development in many plant pathogens (FRAC 2018a). Since 1998, when QoI resistance was first identified in isolates of *Blumeria graminis* (DC.) E.O. Speer f. sp. *tritici* (Bartlett et al. 2002), practical resistance has been reported in more than 50 species representing 35

genera of both fungi and oomycetes (FRAC 2018b). The mechanism of resistance to the QoI fungicides has typically been observed as a nucleotide point mutation in the cytochrome *b* gene that leads to an amino acid substitution at one of three positions in the encoded protein (Fernández-Ortuño et al. 2008). A change from glycine to alanine at position 143 (G143A) results in complete resistance and has been associated with complete control failures (Gisi et al. 2002). Partial resistance has been observed when substitutions of phenylalanine for leucine at position 129 (F129L) or glycine for arginine at position 137 (G137R) occur (Fernández-Ortuño et al. 2008; Gisi et al. 2002; Sierotzki et al. 2007). Cross resistance is frequently observed among members of this class of chemicals, such that isolates resistant to one QoI active ingredient are also resistant to all QoI active ingredients (Fernández-Ortuño et al. 2008; Sierotzki et al. 2000). An additional mechanism of resistance to the QoI fungicides may occur through the activation of an alternative oxidase (AOX) respiration pathway (Fernández-Ortuño et al. 2008; Wood and Hollomon 2003). When this pathway is activated, mitochondrial electron transfer changes course and bypasses the QoI binding site, providing considerably less energy (approximately 40%) to the pathogen (Wood and Hollomon 2003). As a result, this rescue mechanism has a low efficiency of energy conversion and does not support energy-demanding processes such as conidial germination and host penetration that are important for infection (Fernández-Ortuño et al. 2008). This alternative pathway has been shown to limit curative QoI effectiveness in planta with established infections, but for the most part AOX prevents QoI inhibition of spore germination in vitro (Fernández-Ortuño et al. 2008; Miguez et al. 2004; Wood and Hollomon 2003). Salicylhydroxamic acid (SHAM) and propyl gallate (PG) are typical inhibitors of AOX that are often used during in vitro studies of QoI sensitivity (Wood and Hollomon 2003).

A recent partial characterization of the *V. effusa* cytochrome *b* gene revealed the presence of a group I intron directly downstream of the glycine residue at position 143 (Standish et al. 2016). This finding indicates a reduced risk of QoI fungicide resistance, because a mutation in codon 143 would prevent the correct excision of the intron and lead to a defective protein (Cech 1988; Lambowitz and Belfort 1993; Vallières et al. 2011). Practical evidence of this reduced risk of resistance has been observed in field studies in which disease control from azoxystrobin was not compromised despite 20 consecutive applications over 2 years (Standish et al. 2018). These

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fungicides have been used on pecan since the late 1990s, but practical resistance has never been reported. However, efforts to accurately monitor QoI sensitivity have been hindered by the lack of a reliable screening method, because both SHAM and PG are fungitoxic to *V. effusa* in vitro (Seyran et al. 2010a, b). Additionally, a single nucleotide polymorphism (G→A) was recently identified in the first position of codon 137, leading to a novel amino acid substitution from glycine to serine (GGT→AGT; G137S) (Standish et al. 2016). Thus, the objectives of this study were to (i) quantify QoI sensitivity in *V. effusa* using a detached leaf assay and (ii) determine the effects of the G137S amino acid substitution on QoI sensitivity in isolates of *V. effusa*.

Materials and Methods

Isolate collection and preparation. Samples of pecan leaves with actively sporulating pecan scab lesions were collected from two unmanaged baseline orchards in 1997 (orchards with no known history of fungicide exposure), eight managed commercial orchards in 2014, and an experimental orchard with a long history of fungicide use in 2014, 2016, and 2017. Monoconidial isolates of *V. effusa* ($n = 59$) (Table 1) were established from individual leaf lesions following two different protocols, one described by Reynolds et al. (1997) and one outlined by Seyran et al. (2010a).

For samples collected from the two baseline orchards, a single pecan scab lesion was removed from each sampled leaf with a cork borer, and the sporulating side of the lesion was rubbed gently across the surface of 4% water agar (WA) amended with 50 mg/liter each of chloramphenicol, streptomycin sulfate, and tetracycline in Petri plates (Reynolds et al. 1997). The plates were incubated in the dark for 18 to 24 h before individual germinated conidia were transferred to Petri plates containing potato dextrose agar made with the previously stated antibiotic amendments (PDA+) and then incubated at 25°C in the dark to encourage mycelial growth (Reynolds et al. 1997). Colonized agar plugs were transferred to sterile glass tubes and covered with sterile deionized water amended with the previously described antibiotics and 0.1% Tween 20 (SDW+). The glass tubes were stored at room temperature for approximately 20 years before plugs were removed, placed onto PDA+, and incubated in the dark at 25°C to allow for colony growth.

Samples collected from commercial orchards were submitted by pecan growers as part of an orchard-specific fungicide sensitivity monitoring program (Stevenson et al. 2015), whereas experimental orchard samples were collected as part of the aforementioned monitoring program (2014) or from individual trees throughout the course of a separate study (2016 and 2017) (Standish et al. 2018). In both cases, three groups of 15 leaflets were arbitrarily selected from each sample, and conidia were collected by gently pumping SDW+ onto the surface of a sporulating lesion, on each leaflet, with a

micropipette (Seyran et al. 2010a). These bulk conidial suspensions from groups of 15 leaflets were spread across the surface of 2% WA amended with the previously mentioned antibiotics, using a sterile glass rod, and incubated for 24 h at room temperature (23 to 25°C). Similar to the method described by Reynolds et al. (1997), germinated conidia were then transferred to Petri plates containing PDA+ and incubated at 25°C in the dark for 6 to 8 weeks to allow for colonies to develop (Seyran et al. 2010a).

Molecular characterization of *V. effusa* isolates. Genomic DNA was extracted and purified from mycelia of the *V. effusa* isolates ($n = 59$) obtained from samples collected in 1997, 2014, 2016, and 2017 using an UltraClean Microbial DNA Isolation Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. A polymerase chain reaction (PCR) protocol and primers FeCytb_F1 and FeCytb_R1, previously described by Standish et al. (2016), were used to amplify a fragment of the *V. effusa* cytochrome *b* gene, detect mutations at positions 129, 137, and 143, and confirm the presence of two introns within the gene fragment (Standish et al. 2016). PCR products were purified using a QiaQuick PCR Purification Kit (Qiagen, Valencia, CA) and sent to Eurofins MWG Operon LLC (Louisville, KY) for custom sequencing. Geneious version 11.0.5 and ClustalW within MEGA6 were used for analysis of nucleotide sequences (Kearse et al. 2012; Tamura et al. 2013).

Azoxystrobin sensitivity. A detached leaf assay was used to determine the sensitivity of each isolate to azoxystrobin. Two- to 3-year-old pecan trees of cultivar Wichita grafted onto seedling rootstocks were obtained from a commercial nursery. Trees were pruned as necessary and planted in plastic tree pots (25-cm diameter) containing Miracle-Gro potting soil (Scotts Company, Marysville, OH). Except for a dormancy period (November 2017 through March 2018), during which trees were kept outside under ambient conditions, the trees were maintained in a greenhouse, watered biweekly, and fertilized twice per year with a 15N-9P-12K slow-release fertilizer (Osmocote Plus; Scotts Company). Susceptible leaflets were removed from trees and stored at 4°C for up to 1 week before use. Trees were pruned and defoliated periodically to stimulate the production of susceptible leaf tissue for use in the study.

Azoxystrobin (as Abound 2.08F; Syngenta Crop Protection) was suspended in sterile deionized water, and serial dilutions were prepared to obtain final azoxystrobin concentrations of 0.0, 0.0001, 0.001, 0.01, 0.1, or 1 µg of active ingredient per milliliter. The suspensions were applied to the upper surface of the leaflets until runoff using an aerosol propellant atomizer (Crown Spra-Tool; Aervoe Industries, Gardnerville, NV). The leaflets were allowed to air dry before a single leaflet was placed onto the surface of 2% WA amended with 40 µg/ml of benzimidazole for each replicate of each concentration. Leaflets were secured by inserting the leaf base and tip into the agar medium (Cancro 2000).

Conidia for bioassays were produced by placing two hyphal plugs (5-mm diameter) into sterile microcentrifuge tubes containing 1 ml of potato dextrose broth amended with the previously mentioned antibiotics (PDB+) and three sterile 3-mm glass beads. Fungal material was macerated using a bead beating apparatus (Mini-Bead Beater, Biospec, Bartlesville, OK) for 1 min and transferred into tubes containing 6 ml of PDB+. This fungal slurry was mixed with a vortex mixer and transferred onto multiple Petri plates (15- × 60-mm diameter) containing oatmeal agar amended with the previously mentioned antibiotics, in aliquots of 1 ml, and spread across the surface of the media using a sterile glass rod. Plates were placed in an incubator set at 25°C with a 14-h day/night cycle provided by fluorescent light sources. After 10 to 14 days, conidia were harvested by flooding the plates with 3 ml of SDW+. The resulting conidial suspension was centrifuged at 6,500 rpm for 6 min, the supernatant decanted, and the conidial pellet resuspended in 1.5 ml of SDW+ (Cancro 2000). This solution was filtered through two layers of sterile cheesecloth and adjusted to a concentration of 1×10^5 conidia/ml using a hemocytometer (Turechek and Stevenson 1998). A micropipette was used to place 50 µl of the previously prepared *V. effusa* conidial suspension onto each leaflet. The inoculated leaflets were incubated at 25°C in the dark for 48 h (Cancro 2000). Conidial germination

Table 1. Georgia county of origin and number of isolates of *Venturia effusa* collected from pecan and used in this study

Year	County	Orchards ^y	Number of isolates	
			G137S ^z	Collected
1997	Jeff Davis	1	0	9
	Troup	1	0	6
2014	Crisp	2	2	3
	Dougherty	1	0	1
	Lanier	2	0	5
	Peach	1	0	4
	Stewart	2	2	2
	Terrell	2	10	10
	Tift	1	0	6
2016	Tift	1	0	8
2017	Tift	1	0	5
Total			14	59

^y Number of sampled orchards within each county of origin.

^z *V. effusa* isolates with the AGT at codon 137 previously described by Standish et al. (2016).

was determined for 50 conidia per leaflet using a compound microscope ($\times 200$). A conidium was recorded as germinated if the length of the germ tube was at least four times the diameter of the conidium or if an appressorium was visible. For each isolate, the sensitivity tests were conducted twice and in groups of 10 isolates at a time. However, throughout the study, six different isolates of *V. effusa* were included as internal controls and were represented in at least five separate experiments each.

Data analysis. The concentration of azoxystrobin that effectively inhibited conidial germination by 50% (EC_{50}) was estimated for each isolate ($n = 59$) by linearly regressing relative inhibition on $\log_{10}$ -transformed fungicide concentrations using PROC REG in SAS (version 9.4, SAS Institute, Cary, NC). Residuals and quantile plots were examined, and a Shapiro–Wilk test was performed in PROC UNIVARIATE to assess normality of $\log_{10}(EC_{50})$ values for both wild-type isolates ($n = 45$) and those carrying the G137S amino acid substitution ($n = 14$). The $\log_{10}(EC_{50})$ values of wild-type isolates were compared by year of collection (1997, 2014, 2016, and 2017) using a nonparametric Kruskal–Wallis test performed with PROC NPAR1WAY in SAS. Likewise, the distributions of $\log_{10}(EC_{50})$ values of all wild-type isolates were compared with those of the G137S isolates using a nonparametric, two-sided Wilcoxon rank sum test, also performed in SAS. The coefficient of variability of $\log_{10}(EC_{50})$ values, which is the standard error of $\log_{10}(EC_{50})$ values divided by mean of $\log_{10}(EC_{50})$ values, was calculated for the six internal control isolates and used to assess reproducibility of the assay.

Results

Molecular characterization of *V. effusa* isolates. The primers amplified a DNA fragment of approximately 600 bp, and sequence analyses confirmed the presence of a portion of the 1,407-bp intron as well as the presence of the previously described 360-bp intron downstream of position 135 in all 59 isolates. Phenylalanine (F) and glycine (G) were present at positions 129 and 143, respectively, in all 59 isolates. The previously described G137S substitution was confirmed in the cytochrome *b* gene of 14 isolates as a mutation of guanine replaced by adenine in the codon at position 137 (GGT→AGT) (Standish et al. 2016). Partial nucleotide sequences of the cytochrome *b* gene for the 14 *V. effusa* isolates carrying the G137S substitution (ST11A, ST12A, TE9A, TE9B, TE9C, TE9D, TE9E, TE11A, TE11B, TE11C, TE11D, TE11E, CR5A, and CR5B) and four wild-type *V. effusa* isolates (JD-97-20, JD-97-22, TC8, and TC38) were deposited into GenBank (accession nos. MH647746 to MH647763).

Azoxystrobin sensitivity. For each of the six internal control isolates of *V. effusa* examined in at least five experimental repeats of this assay, the mean EC_{50} values ranged from 0.0004 to 0.0138 $\mu\text{g/ml}$, and coefficients of variability ranged from 0.0350 to 0.1080 (Table 2). The mean EC_{50} value for these six isolates was 0.0053 $\mu\text{g/ml}$ with a mean coefficient of variability of 0.0662 (Table 2). All coefficients of variability were less than 0.2, indicating that the results for the isolates were consistent among experimental repeats. Therefore, the mean EC_{50} values for the remaining isolates screened in this study were determined based on combined data from all experimental repeats. Frequency distributions of the mean EC_{50} values were assessed for normality, but for 10 of the 45 wild-type isolates, EC_{50} values could not be estimated (values $< 0.0001 \mu\text{g/ml}$). When those 10 isolates were excluded from the analysis, the assumptions of normality for $\log_{10}(EC_{50})$ values of the remaining isolates were met ($[Pr < W] = 0.7712$). The distribution of mean $\log_{10}(EC_{50})$ values for isolates carrying G137S was also normal ($[Pr < W] = 0.1327$). The mean EC_{50} values for the 59 isolates screened for their sensitivity to azoxystrobin ranged from a minimum of < 0.0001 to a maximum of 0.3047 $\mu\text{g/ml}$ (Table 3). Year of collection was not a significant factor for wild-type isolates ($P = 0.1354$); thus, the baseline isolates included in this study ($n = 15$) were combined with the remaining wild-type isolates ($n = 30$) for further analysis. Mean EC_{50} values for wild-type isolates ranged from < 0.0001 to 0.2007 $\mu\text{g/ml}$, whereas those with G137S ranged from 0.0033 to 0.3047 $\mu\text{g/ml}$ (Fig. 1; Table 3). Overall, isolates with G137S were approximately eight

times less sensitive to azoxystrobin than the wild-type isolates, based on the respective medians, which differed significantly according to the two-sided Wilcoxon rank sum test ($P = 0.0010$) (Table 3).

Discussion

All fungicides labeled for pecan scab management have some inherent risk of resistance, ranging from “low to medium” to “high” (FRAC 2018a). Practical resistance or significant reduction in sensitivity has been documented for the DMIs, methyl benzimidazole carbamates, and organotin (Littrell 1976; Reynolds et al. 1997; Seyran et al. 2010a; Standish et al. 2018; Stevenson et al. 2004, 2015). After nearly two decades of use, practical resistance to the QoIs has never been reported despite their high risk for resistance to develop. Similarly, shifts in QoI sensitivity have never been reported; however, the fungitoxic effects of SHAM and PG on *V. effusa* in vitro have thwarted monitoring efforts. The recent discovery of a single nucleotide polymorphism in the first position of codon 137 of the *V. effusa* cytochrome *b* paired with the inability of traditional in vitro bioassays (i.e., spore germination/mycelial growth assays) to accurately quantify QoI sensitivity underscore the importance of our efforts to develop this alternative screening method. In this study, we developed a detached leaf assay for use in quantifying sensitivity of *V. effusa* isolates to azoxystrobin without the need for an AOX inhibitor, because the alternative respiration pathway is not known to interfere with QoI activity against conidial germination in planta. This reproducible method has a known level of precision and was used to generate the first frequency distribution of *V. effusa* sensitivities to a member of this important chemical class. Although labor intensive and time consuming, the assay and the results generated herein may be used in the future to detect potential shifts in sensitivity to azoxystrobin within *V. effusa* populations. Additionally, this is the first characterization of sensitivity to a QoI fungicide for isolates of *V. effusa* or any phytopathogen carrying the G137S amino acid substitution reported previously (Standish et al. 2016).

The distribution of mean EC_{50} values calculated for all 59 isolates assayed in this study was unimodal and slightly skewed to the right, with 10 isolates that exhibited EC_{50} values less than the lowest concentration tested (0.0001 $\mu\text{g/ml}$) (Fig. 1). Isolate sensitivity did differ significantly when comparing wild-type and G137S isolates. Based on the respective median EC_{50} values, G137S isolates were approximately eight times less sensitive than the wild-type isolates; however, there was some overlap between the respective sensitivity distributions (Fig. 1). This reduction in sensitivity of G137S isolates of *V. effusa* is consistent with the reduction in sensitivity observed in isolates of *Pyrenophora tritici-repentis* (Died.) Dreschler exhibiting both F129L and G137R (Sierotzki et al. 2007). Unlike the complete resistance associated with the G143A substitution, the partial resistance reported in isolates with the F129L substitution does not appear to have the same level of impact on fungicide efficacy (Pasche et al. 2005; Sierotzki et al. 2007). A study examining the effects of azoxystrobin on disease caused by F129L isolates of *Alternaria solani* Sorauer ($n = 5$) revealed that significant reductions in efficacy may occur, although this was not consistent for all isolates tested

Table 2. Reproducibility of effective concentration of azoxystrobin at which conidial germination was inhibited by 50% (EC_{50} values) determined for six internal control isolates of *Venturia effusa* using a detached leaf assay

Isolate	Azoxystrobin EC_{50} ($\mu\text{g/ml}$)			CV ^z
	Mean	Median	Range	
JD-97-20	0.0024	0.0029	0.0009–0.0032	0.0366
JD-97-9	0.0004	0.0003	0.0001–0.0007	0.0350
P16AT3	0.0138	0.0084	0.0014–0.0491	0.1080
P16AT4	0.0025	0.0018	0.0004–0.0059	0.0685
TC8	0.0012	0.0004	0.0001–0.0042	0.0851
TE9B	0.0117	0.0071	0.0043–0.0279	0.0642
Mean	0.0053	0.0035	0.0012–0.0152	0.0662

^z CV = coefficient of variability expressed as the absolute value of [standard error of $\log_{10}(EC_{50})$ values]/[mean of $\log_{10}(EC_{50})$ values].

(Leiminger et al. 2013). Additionally, field studies in Minnesota and North Dakota examining control of early blight of potato have shown that QoI fungicides have not performed better than older, multisite fungicides (i.e., chlorothalonil or mancozeb) when the pathogen population is dominated by F129L isolates (95.7% of 1,212 isolates in Minnesota and 99.3% of 1,300 isolates in North Dakota, from 2002 to 2006) (Pasche and Gudmestad 2008). To date, studies testing QoI efficacy on trees inoculated with G137S isolates of *V. effusa* have not been conducted and merit further examination.

In both the case of *P. tritici-repentis* (Sierotzki et al. 2007) and the isolates of *V. effusa* used in this study, the amino acid substitution at residue 137 was caused by a change in the first nucleotide of the codon (GGT→AGT). In nonphytopathogenic organisms, single nucleotide changes leading to G137V or G137E substitutions have been linked with a reduced respiratory capacity (Brasseur et al. 1996; Fisher and Meunier 2001). Thus, a mutation at this position may result in a potential fitness

penalty in the pathogen that could prevent its long-range distribution or ability to dominate the overall population (Brasseur et al. 1996; Fisher and Meunier 2001; Gisi et al. 2002). The G137S isolates identified in this study were obtained from samples collected from four managed pecan orchards in three Georgia counties; these samples had 100% frequency of G137S isolates (Crisp County, 2/2; Stewart County, 2/2; Terrell County, 5/5 and 5/5, respectively) (Table 1). These isolates represent approximately 24% of all isolates assayed in this work, although selection for use herein was based on presence or absence of the mutation and is not likely representative of the overall population. In our previous work, based on extensive state-wide sampling, seven of 125 isolates carried G137S (approximately 6%), which is likely to be a more accurate representation of its prevalence across Georgia pecan orchards (Standish et al. 2016). However, the prevalence of G137S in individual pecan orchards is not known but may lead to an impact on efficacy as in *A. solani* populations dominated by the F129L substitution (Pasche and Gudmestad 2008).

Previous research characterizing a portion of the *V. effusa* cytochrome *b* gene revealed a group I intron directly downstream of position 143 in 125 isolates (Standish et al. 2016). Owing to the mechanism by which group I introns are removed from mRNA transcripts, a nucleotide mutation like that which causes the G143A substitution would be lethal as the result of a nonfunctional protein, and such mutants would disappear from the population (Cech 1988; Lambowitz and Belfort 1993; Vallières et al. 2011). A 2-year field study of fungicide efficacy for pecan scab control provided practical evidence of this expected reduced risk of resistance (Standish et al. 2018). After 10 consecutive applications per season, Abound 2.08F (azoxystrobin) and Abound 2.08F plus Orius 3.6F (azoxystrobin plus tebuconazole) resulted in less disease than most other

Table 3. Median and range of effective concentration of azoxystrobin at which conidial germination was inhibited by 50% (EC₅₀ values) for isolates of *Venturia effusa*

Isolates	Codon 137 ^y	Number of isolates	EC ₅₀ (µg/ml)	
			Median	Range
Wild type	GGT	45	0.0023 b ^z	<0.0001–0.2007
G137S	AGT	14	0.0178 a	0.0033–0.3047
Combined	...	59	0.0043	<0.0001–0.3047

^y Nucleotide sequence of codon 137 of the *V. effusa* cytochrome *b* gene.

^z Different letters indicate significant differences between medians based on the Wilcoxon rank sum test ($P = 0.0010$).

Fig. 1. Frequency distribution of effective concentration of azoxystrobin at which conidial germination was inhibited by 50% (EC₅₀ values) for isolates of *Venturia effusa* carrying sequences coding for either glycine (wild type; top) or serine (G137S; bottom) at position 137 of the cytochrome *b* gene. Bars are positioned on the midpoint of each respective histogram bin. Vertical dashed lines represent median log₁₀EC₅₀ values. The asterisk (*) indicates 10 isolates with EC₅₀ values less than the lowest concentration tested in this study.

fungicide treatments included in the study (Standish et al. 2018). Several of the isolates assayed in the present study were collected from the experimental orchard where these products were tested in 2016 and 2017 (Tift County; Table 1). The mean EC₅₀ values for these isolates were similar to those of the baseline isolates collected in 1997 (Table 1), despite the consistent selection pressure exerted on the population. Additionally, none of those isolates carried the G137S substitution. As mentioned previously, there have been no reports of practical resistance to QoI fungicides in the pecan scab pathogen even though several fungicides in this group have been available for pecan since the late 1990s; however, monitoring sensitivity had been difficult until now.

In developing this detached leaf assay, we now have a reproducible method for quantifying QoI sensitivity in *V. effusa*. The results generated here are useful for future monitoring efforts to identify shifts in sensitivity when or if they occur. Isolates with the G137S amino acid substitution were less sensitive than the wild-type isolates, but the impact of these mutants on QoI efficacy in the field is currently unknown. Based on the sequence results, all 59 isolates tested in this study possessed the group I intron downstream of position 143. This indicates that an amino acid substitution at position 143 would be lethal and provides further evidence of a reduced risk of QoI resistance in *V. effusa* (Standish et al. 2016). However, resistance may still develop after repeated or long-term exposure if intron-lacking genotypes occur and are selected for, or additional and as-of-yet-unknown mechanisms of resistance are identified (Fernández-Ortuño et al. 2008; Vallières et al. 2011).

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