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Detection of the G143A Mutation that Confers Resistance to QoI Fungicides in *Alternaria tomatophila* from Tomatoes

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ABSTRACT

During the 2014 tomato growing season in Indiana the control of early blight caused by *Alternaria* spp. was difficult to achieve. QoI fungicides are still part of the spray programs designed to control early blight and other tomato diseases, despite the fact that *Alternaria* isolates less sensitive to QoI fungicides have been reported since 2007 in tomato producing areas in the United States. The cytochrome *b* gene mutation associated with QoI sensitivity shift is the F129L mutation (substitution of phenylalanine for leucine at position 129). Isolates of *Alternaria* spp. were collected from tomato fields in Indiana and their response and resistance mechanism to the QoI fungicides azoxystrobin and famoxadone was determined. Isolates were identified as *A. tomatophila* based on conidial morphology and molecular tools. It was found that most of the isolates had the G143A mutation (glycine for alanine at position 143). The detection of G143A mutation explains the very low levels of early blight control obtained with the QoI fungicides. To our knowledge, this is the first report of the detection of QoI resistant isolates of *A. tomatophila* having the G143A mutation.

INTRODUCTION

Tomato production in the United States is a US $2 billion industry. Seventy five percent of the total area planted is on processing tomatoes. The biggest producer of fresh tomatoes is California harvesting about 94% of the total area planted. In the Midwest processing tomatoes are grown in Indiana, Ohio, and Michigan and these states account for most of the remaining production (Wells, 2016). Early blight is the most aggressive diseases affecting the production of processing tomatoes in the Midwest states, but this disease in California is of minor importance (Jones et *al*. 2014).

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In the United States, early blight in tomatoes is mainly caused by *Alternaria solani*. Another *Alternaria* species, *A. tomatophila* has also been reported but at a lower frequency (Jones et *al.* 2014). The chemical control of early blight mainly relies on the Quinone outside Inhibitors (QoI) fungicides azoxystrobin and famoxadone. These fungicides have been usually sprayed in combination with chlorothalonil or mancozeb. After claims of reduced control with azoxystrobin and famoxadone in some areas the succinate dehydrogenase inhibitor (SDHI) fungicides are starting to be introduced for the control of this disease.

The mode of action of QoI fungicides (FRAC group 11) is the inhibition of mitochondrial respiration by blocking the electron flow from cytochrome *bc1* to the other proteins in the respiratory chain thus preventing ATP formation. The first case of resistance in *A. solani* isolates causing early blight of tomatoes to QoI fungicides was detected in Michigan in 2005 and then in Indiana in 2006 (Olaya et *al*. 2007). The cytochrome *b* (*cyt b*) mutation conferring resistance to QoI fungicides in *A. solani* was identified as the F129L. In 2005, a reduction on early blight control on tomatoes was reported in New York state but there was no mention of any mutation involved in resistance (Zitter et *al*. 2007). In potatoes, *A. solani* isolates resistant to QoI fungicides were identified after the 2002 season in North Dakota and Wisconsin (Pasche et *al*. 2004; Rozenzweig et *al*. 2008).

In this study, we report for the first time QoI fungicide resistance in *A. tomatophila* isolates collected from open commercial fields of processing tomatoes in the USA. In addition we report for the first time the detection of the G143A mutation in the *cytb* gene that confers resistance to QoI fungicides in *A. tomatophila*.

MATERIALS AND METHODS

Isolates collection:

Alternaria isolates were collected from tomato commercial fields in Indiana where the QoI fungicides did not control the disease at satisfactory levels. Maintenance of the isolates and production of conidia was conducted on clarified V8 juice agar.

Sensitivity test:

The sensitivity of each isolate to azoxystrobin and famoxadone was determined by comparing the conidial germination of each isolate on water agar (WA) plates amended or not with the fungicides. The final concentrations of azoxystrobin and famoxadone in the media were 0, 0.001, 0.01, 0.1, 1.0, and 10.0 mg/L. In addition, the WA medium was amended with 100 mg/L of Salicylhydroxamic acid (SHAM) dissolved in methanol in order to inhibit the alternative oxidase respiratory pathway (Olaya et *al.* 1998). A conidial suspension was prepared and adjusted to 1 x 10^4 conidia per milliliter and 50 µl sample were spread on the surface of plates amended or not with fungicide. The plates were incubated at 22-24 °C for 4-6 hours. A conidium was rated as germinated if a normally developing germ tube was at least

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.

Alternaria species identification

A. solani and *A. tomatophila* are morphologically very similar species and closely related large-spored *Alternaria* species. Morphological characterization of the conidia was conducted by measuring the length and width of the conidia and the length of the beak and branching of three isolates of each *Alternaria* species.

Three different molecular markers were used to confirm the *Alternaria* species: a) *Alternaria* major allergen *Alt* a1; b) calmodulin genes; c) SDH genes. For the molecular species identification a comparison of the PCR bands was obtained based on species-specific primers for *A. solani* and *A. tomatophila* already described by Gannibal et *al*. 2014. These primers amplify fragments of the *Alternaria* major allergen *Alt* a1 and calmodulin genes. The *A. solani*-specific primers OAsF7 (5′-CGACGAGTAAGTTGCCCTCA-3′) and OAsR6 (5′-TGTAGGCGTCAGAGACACCATT-3′) give an amplicon of 164 bp for *A. solani* and doesn't give an amplicon for *A. tomatophila.* The *A. tomatophila*-specific primers OAtF4 (5′-TGCGGCTTGCTGGCTAAGGT-3′) and OAtR2 (5′-CAGTCGATGCGGCCGTCA-3′) give an amplicon of 483 bp for *A. tomatophila* and doesn't give an amplicon for *A. solani.* The thermal conditions for the amplification with combination of primers OAsF7 /OAsR6 and OAtF4/OAtR2 were performed following the protocol of Gannibal et *al*. (2014).

Three different subunits of the succinate dehydrogenase gene (*sdhB, sdhC* and *sdhD*) were also amplified and sequenced to distinguish *A. solani* from *A. tomatophila*. The primers described in Table 1 were used and the thermal conditions were the following: 95°C for 3 min, followed by 30 cycles at 95°C for 25 sec, 60 °C for 25 sec, 72°C for 1 min (2 min was used for *sdhB*), and a final extension step at 72°C for 7 min. The sizes of the amplicons for *sdhB, sdhC and sdhD* were 1060 bp, 622 bp and 633 bp, respectively. To get a better sequence comparison, the three SDH subunits were also sequenced for *A. alternata* from USA and from Europe and for *A. brassicae* from Europe.

Table 1 Primer sequences used to amplify the SDH B, C, D and *CytB* genes

Primer name	**Sequence (5`-3`)**
Alt_*sdhB*_f6	GCGCTTTCACTCGTCTGGCTACCC
Alt_*sdhB*_rv4	CCATGCTCTTCTTGATCTCCGC
Alt_*sdhC*_f1	ATGGCTTCTCAGCGGGTATTTCAGC
Alt_*sdhC*_r2	GGTGTAGTAAAGGCTGAATGCGACGG
Alt_*sdhD*_f1	ATGGCCTCCGTCATGCGTCC
Alt_*sdhD*_r1	TATGCGTGCCACAACCTCGCGACG
AS-5F	AGAACTCTAGTATGAACTATTGG
Asint4dr	TCATTCTGGCACGATAGCTG

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Mechanism of QoI fungicide resistance:

To identify the possible mutations involved in the QoI resistance, a fragment covering the amino acids in position 129 and 143 was sequenced. RNA from *A. solani* and *A. tomatophila* were reverse transcribed to cDNA and then amplified with primer combination AS-5F/ Asint4dr (Grasso et *al*. 2006) described in Table 1. The reverse transcription and the amplification were performed using the Access RT-PCR system (Promega, USA) following the instructions provided by the manufacturer. Sequencing of the cDNA was performed following the BigDye Terminator v3.1 protocol (Applied Biosystems). In order to check the presence/absence of an intron after the position codon 143, *cytb* gene amplification was performed using genomic DNA (gDNA) with the same primer combination AS-5F/ Asint4dr (Table 1). The amplification for both *Alternaria* species was performed using the Phusion High Fidelity DNA polymerase protocol (New England Biolabs, Inc (NEB)), following the instructions provided by the manufacturer. The thermal conditions consisted of: 95°C for 1 min, followed by 35 cycles at 95°C for 30sec, 60 °C for 30sec, 72°C for 5 min and a final extension step at 72°C for 5 min. The expected size in gDNA of *A. solani* with this combination of primers was about 6800 bp.

Pathogenicity and virulence of the Alternaria isolates and control with QoI fungicides:

One isolate of *A. solani* (sensitive to QoI fungicides) and two isolates of *A. tomatophila* (one sensitive and one resistant to QoI fungicides) were selected to inoculate tomato plants treated or not with azoxystrobin or famoxadone at a rate of 55 g ai/ha.

RESULTS

A total of 17 large-spored *Alternaria* isolates were collected from processing tomato fields in Indiana in 2014. All these isolates were identified as *A. tomatophila* with different molecular markers (*Alternaria* major allergen *Alt* a1, calmodulin genes and *Sdh* genes). The sequences obtained from the *sdh* gene subunits B, C and D from *A. solani* reference isolates from USA and Europe showed several single nucleotide polymorphisms (SNPs) that allowed to differentiate them from *A. tomatophila*.

Eight different SNPs in the coding region and two in the non-coding region of the *sdh*D gene (Table 2) were identified to differentiate *A. solani* from *A. tomatophila*. One of these SNPs (at amino acid position at codon 48) was non-synonymous and encoded for 2 different amino acids (valine for *A. solani* and isoleucin for *A. tomatophila*) (Table 2). One SNP in the *sdhB* and five in the *sdhC* were found to differentiate *A. solani* and *A. tomatophila* (data not shown). The sequence analysis of the *sdh* gene subunits showed that *A. solani* and *A. tomatophila* are more closely related to each other than any other *Alternaria* species (*A. alternata and A. brassicae*). The main SNPs in *sdhD* that differentiate *A. solani* from *A. tomatophila* are described in Table 2.

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Table 2 The ten SNPs in the coding and non-coding regions of the *sdhD* gene that separate *A. solani* from *A. tomatophila*.

Amino acid	Amino acid position	Polymorphism	Alternaria specie	Location
T	28	ACT ACC	*A. solani* *A. tomatophila*	Exon
S	46	TCA TCC	*A. solani* *A. tomatophila*	Exon
V I	48	GTC ATC	*A. solani* *A. tomatophila*	Exon
P	67	CCA CCC	*A. solani* *A. tomatophila*	Exon
P	80	CCC CCA	*A. solani* *A. tomatophila*	Exon
I	106	ATT ATC	*A. solani* *A. tomatophila*	Exon
L	126	CTA CTC	*A. solani* *A. tomatophila*	Exon
L	130	CTC CTG	*A. solani* *A. tomatophila*	Exon
	422 (nucleotide position)	C T	*A. solani* *A. tomatophila*	Intron
	446 (nucleotide position)	T C	*A. solani* *A. tomatophila*	Intron

Table 3 Summary of sensitivity towards QoI fungicides of selected isolates, the *cyt b* characterization and species identification based on the presence/absence of PCR amplicons (Yes: amplicon present. No: amplicon absence)

				Cytb characterization			Species ID	
Isolate Number	**Fungal species**	**AZO EC_{50}**	**FMOX EC_{50}**	**CytB F129L**	**CytB G143A**	**Band size (gDNA) primers As-5f and Asint4dr**	**Primers OAsF7-OAsR6**	**Primers OAtF4-OAtR2**
14-356	*A. tomatophila*	0.131	0.435	F	G	~ 4800 bp	No	Yes
14-358	*A. tomatophila*	5.746	>10	F	A	~ 4800 bp	No	Yes
14-359	*A. tomatophila*	2.731	>10	F	A	~ 4800 bp	No	Yes
14-361	*A. tomatophila*	2.276	8.153	F	A	~ 4800 bp	No	Yes
14-364	*A. tomatophila*	2.383	>10	F	A	~ 4800 bp	No	Yes
14-365	*A. tomatophila*	2.962	>10	F	A	~ 4800 bp	No	Yes
14-366	*A. tomatophila*	2.039	>10	F	A	~ 4800 bp	No	Yes
14-368	*A. tomatophila*	3.813	>10	F	A	~ 4800 bp	No	Yes
14-369	*A. tomatophila*	2.929	>10	F	A	~ 4800 bp	No	Yes
14-370	*A. tomatophila*	7.232	>10	F	A	~ 4800 bp	No	Yes
14-374	*A. tomatophila*	4.905	>10	F	A	~ 4800 bp	No	Yes
14-375	*A. tomatophila*	3.251	>10	F	A	~ 4800 bp	No	Yes
14-377	*A. tomatophila*	2.437	>10	F	A	~ 4800 bp	No	Yes
11-288*	*A. tomatophila*	0.034	0.021	F	G	~ 4800 bp	No	Yes
11-111**	*Alternaria solani*	0.013	0.012	F	G	~ 6800 bp	Yes	No
99-35**	*Alternaria solani*	0.037	0.024	F	G	~ 6800 bp	Yes	No
99-25**	*Alternaria solani*	0.044	0.023	F	G	~ 6800 bp	Yes	No

** A. tomatophila reference isolate; ** A. solani reference isolates*

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The *Alternaria* spp. identification results using specific primers OAsF7/OAsR6 (*A. solani*) and OAtF4/OAtR2 (*A. tomatophila)* are reported in Table 3. The results from the three molecular markers confirm that the 17 isolates collected in Indiana are *A. tomatophila*. From these 17 isolates the G143A mutation in the *cytb* was found in 16 isolates, while the mutation was absent one isolate. All the 17 isolates showed the wild type allele F129 allele (phenylalanine (F) at amino acid position 129). The amplification of the complete *cytb* gene using gDNA from *A. tomatophila* with primer set AS-5F/Asint4dr showed a smaller amplicon (around 4800bp) than the one in *A. solani (*around 6800 bp) (Table 3).

Sixteen of the 17 isolates of *A. tomatophila* were found to be resistant to the QoI fungicides azoxystrobin and famoxadone using the conidia germination assay (Table 3). The QoI sensitive *A. tomatophila* had an EC_{50} value for azoxystrobin (AZO) and famoxadone (FMOX) of 0.034 and 0.021 mg/L, respectively. The *A. solani* reference isolates were sensitive to both QoI fungicides. Conidia of *A. tomatophila* had on average a smaller and thinner body (85 x 23 μm) in comparison to *A. solani* (109 x 34 μm). The average length of the beak of *A. tomatophila* conidia was longer (120 μm) compared to the ones of *A. solani* (79 μm). The total length of the conidia (body plus beak) was on average slightly longer for *A. tomatophila* (205 μm) in comparison to *A. solani* (189 μm).

The typical number of branches of the conidia beak was one or two in *A. solani* and one to three in *A. tomatophila*. The *A. tomatophila* and *A. solani* isolates were all pathogenic and virulent to tomato plants, but the *A. tomatophila* isolates showed to be more aggressive than the *A. solani* isolates (data not shown). The QoI sensitive isolates of *A. solani* and *A. tomatophila* were fully controlled with azoxystrobin and famoxadone, but the resistant *A. tomatophila* isolates were not controlled by any of the two QoI fungicides tested (data not shown).

DISCUSSION

QoI fungicides are part of the spray programs designed to control early blight and other processing tomato diseases. The early blight disease outbreak in 2014 was difficult to control using QoI fungicides (azoxystrobin or famoxadone) in Indiana, USA. The sampled *Alternaria* were identified as *A. tomatophila* based on conidial morphology and molecular tools. The SNPs in the *sdh* genes (especially in the *sdhD* subunit) together with the presence or absent of the amplicons approach using the *Alternaria* major allergen *Alt* a1 and calmodulin genes (Gannibal et *al*. 2014) can be used asrobust molecular tools to differentiate between *A. solani* and *A. tomatophila*.

The resistance allele involved in the QoI resistance of *A. tomatophila* isolates is the mutation in the *cytb* gene known as the G143A (glycine for alanine substitution at position 143). A different *cytb* mutation, resulting in F129L, has been previously described to confer resistance to QoI fungicides in *A. solani* from processing tomatoes (Olaya et *al*. 2007). The difference in size of around 2000 bp of the amplified *cytb* gene fragment between *A. tomatophila* (smaller amplicon of around 4800 bp) and *A. solani (*bigger amplicon of around 6800 bp) (Table 3) confirm the absence of an intron right after the position 143 of the *cytb* gene in *A. tomatophila.*

The intron absence allowed the G143A mutation to evolve in *A. tomatophila* and its presence restrict the occurrence of this mutation in *A. Solani* (Grasso et *al*. 2006).

The detection of G143A mutation in most of the isolates from Indiana explaines the very low levels of early blight control obtained with the QoI fungicides. In different plant pathogenic fungi, the G143A mutation has been recognized to confer higher levels of QoI resistance in comparison to the F129L mutation (Grasso et *al*. 2006; Rozenzweig et *al*. 2008). To our knowledge, this is the first report of QoI resistant isolates of *A. tomatophila* based on the G143A mutation.

The *A. tomatophila* isolates showed to be more pathogenic and virulent to tomato plants than the *A. solani* isolates. It was demonstrated that the *A. tomatophila* QoI resistant isolates are not controlled with azoxystrobin or famoxadone. These results indicate that resistant isolates carrying the G143A mutation are fit with no changes in their pathogenicity and virulence to tomato plants treated or not with QoI fungicides. Future QoI resistance monitoring studies conducted on early blight of tomatoes should consider diagnosis of the underlying *Alternaria* species causing the disease and the identification of the correct mutation in the *cytb* gene causing resistance.

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