G143A MUTATION IN THE *CYTOCHROME B* GENE DETECTED FROM *CORYNESPORA CASSIICOLA* ISOLATES IN ALABAMA

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Abstract

Corynespora cassiicola, the causal agent of target spot disease, is a fungal pathogen with increasing importance in cotton and soybean producing countries. Quinone outside inhibitors (QoI) is one of the most important classes of fungicides widely used for disease management of foliar diseases in field crops; however, there are several reported cases of *C. cassiicola* isolates from tomato, cucumber, and soybean resistant to these fungicides. QoI-resistance has been detected in the *cytochrome b* (*cytb*) gene which a nucleotide substitution in a specific codon, such as G143A, F129L, and G137R, confer resistance to QoI fungicides. Therefore, we investigated the occurrence of a point mutation in the cytb gene associated with QoI-resistance of 12 isolates of *C. cassiicola* from cotton and soybean collected in Alabama. Multiple alignment analysis revealed a mutation at position 143 (G143A) in four isolates from soybean plants. The mutation changed the codon 143 (GGT to GCT), resulting in an amino acid change from glycine to alanine (G143A), which is known to be associated with QoI-resistance. The G143A mutation was not found on isolates from cotton plants. Other mutations, F129L and G137R, were not found in our isolates. The present study is the first report of the occurrence of the G143A mutation associated with *C. cassiicola* in the U.S. The dynamic of mutations associated with fungicide resistance in *C. cassiicola* should be monitored for management of resistance to *C. cassiicola* in the U.S.

Introduction

Corynespora cassiicola (Berk. & Curt.) C.T. Wei is a widespread plant pathogenic fungus that causes target-shaped necrotic spots on plant leaves and on stems, roots, flowers, and fruits and has been recorded worldwide on up to 400 plant species (Farr and Rossman 2017). The foliar phase of the disease is characterized as small circular spots, varying between 2 mm and 10 mm. Well-developed lesions are necrotic and show typical “target spot” symptoms, with some depression at the center of the lesion. In severe cases of infection, the lesions coalesce and the leaves show severe necrosis followed by complete premature senescence and death of the leaf (Galbieri et al., 2014). The disease is known as Corynespora leaf spot or Target spot on cotton and soybean. In the Southeastern U.S. genetically distinct populations of *C. cassiicola* were found causing target spot epidemics on cotton and soybean (Sumabat et al. 2018).

Disease management has been a concern in other countries due to increasing occurrence of target spot (Godoy et al., 2015). Severe disease symptoms and significant yield losses can occur when the pathogen is not properly controlled (Hagan and Sikora, 2012; Koenning et al., 2006). Yield losses up to 400 lb./acre of seed have been estimated for cotton (Bowen et al., 2018), and up to 900 lb./acre of yield losses for soybean (Faske, 2016). Fungicides have been a crucial tool in disease management; however, there are reported cases of *C. cassiicola* isolates from tomato, cucumber, and soybean resistant to specific fungicides (Fungicide Resistance Action Committee – FRAC, www.frac.info/publications. Methyl Benzimidazole Carbamates (MBC, FRAC code 1) (Date et al., 2004; Xavier et al., 2013; Avozani et al., 2014; Teramoto et al., 2017), Succinate-dehydrogenase inhibitors (SDHI, FRAC code 7) (Miyamoto et al., 2009, 2010) and Quinone outside Inhibitors (QoI, FRAC code 11) are fungicides included in those reported cases of resistance.

Corynespora cassiicola is considered a high-risk pathogen for development of fungicide resistance (FRAC 2019), and mutations associated with QoI-resistance have been detected in the *cytochrome b* (*cytb*) gene based on three amino acid substitutions: G143A, F129L, and G137R (Duan et al. 2019; FRAC 2019). G143A mutation has been characterized for *Cercospora sojina* (Mathew et al. 2019) in the U.S. but not for *C. cassiicola*.

Due to the intensive use of fungicides to control cotton and soybean diseases, QoI-resistant populations of *C. cassiicola* might be present in the United States. For this reason, the objective of this study was to assess the occurrence of point mutations in the *cytb* gene associated with QoI-resistance from Alabama isolates of *C. cassiicola*.

Materials and Methods

Isolates

During 2017/18, isolations of *C. cassiicola* were carried out using direct isolation in which small amounts of mycelia and conidia from symptomatic lesions on leaves of cotton and soybean were directly transferred onto potato dextrose agar (PDA) containing 0.005% kanamycin. PDA plates were sealed and incubated at room temperature (RT, 25 ± 2°C) for mycelial growth. Pure colonies were transferred to potato dextrose agar (PDA; DIFCO Laboratories) plates to establish the *C. cassiicola* Alabama collection. All isolates were identified as *C. cassiicola* based on conidiophore and conidia morphology (Ellis, 1971) and ITS sequencing (ITS1/ITS4). All *C. cassiicola* isolates were stored at -80˚C.

DNA extraction

Mycelial plugs of 12 selected isolates were placed over a cellophane membrane onto a fresh PDA plate (Cassago et al., 2002). After 10 days of incubation at RT, mycelia were harvested, and total DNA were extracted from each isolate using a ZR Fungal/Bacterial MiniPrep™ kit from Zymo Research (California, USA). Extracted DNA concentrations were evaluated using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA) and stored at -20˚C prior to use.

Detection of the point mutation on *cytb*

To identify nucleotide point mutation on *cytb* gene, fragments were amplified from total DNA using PCR primers CcCytb-F (5'-GCGAATTCCTATTTAGTTGATTC-3') and CcCytb-R (5'-GGTTACCTGATCCAGCTGTATC-3') (Duan et al. 2019). PCR was performed on 20 ng/µL of *C. cassiicola* genomic DNA. A 50-µl reaction mix was prepared for each isolate containing 2 µl of each purified DNA sample, 1 µl of forward primers, 1 µl of reverse primers, 21 µl of deionized water, and 25 µl of JumpStart *Taq* ReadyMix (Sigma-Aldrich, St. Louis, MO, USA). Reactions (50 µl) were pipetted into 8-tube strips, and PCR was conducted. DNA amplification was carried out in a MultiGene DNA thermal cycler (Labnet International; Edison, NJ) with a program consisting of initial denaturation for 5 min at 94˚C; followed by 35 cycles of 1 min at 94˚C, 1 min at 55˚C, and 2 min at 72˚C; and a final cycle of 5 min at 72˚C. Amplification products were visualized under UV light on 1.0% agarose gels stained with GelRed® Nucleic Acid Gel Stain (Biotium Inc., Fremont, CA, USA) to detect the *cytb* gene. Purified PCR products were Sanger sequenced by Eurofins MWG Operon LLC (Louisville, KY), and nucleotide sequences were edited and aligned using BioEdit Alignment Editor (Tom Hall, Ibis Biosciences). QoI-sensitive (C6-2) and QoI-resistant (ST-20S-1) sequences of *C. cassiicola* (Ishii et al. 2007) were included to illustrate the nucleotide point mutation. Sequences were deposited in GenBank under accession numbers MN564884-MN564895.

Results and Discussion

The amplification on agarose gel of PCR products from the genomic DNA of six *C. cassiicola* isolates from cotton (BRW03, MAC01, FHP01, FHP22, HSV01, and HSV12) and six *C. cassiicola* isolates from soybean (LIM02, LIM13, LIM14, ELM04, ELM06, and ELM07) was successful using primers previous published by Duan et al. (2019) (Fig 1).

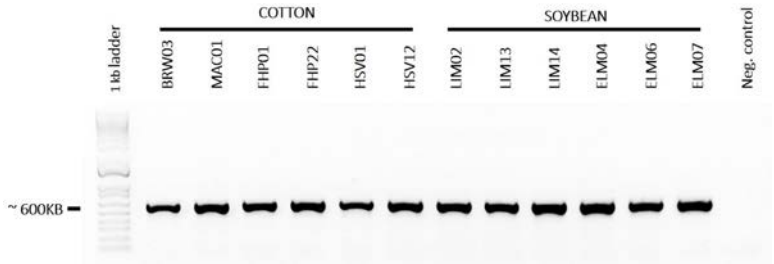

Figure 1. Agarose gel of PCR products amplified from genomic DNA of six *C. cassiicola* isolates from cotton (BRW03, MAC01, FHP01, FHP22, HSV01 and HSV12) and six *C. cassiicola* isolates from soybean (LIM02, LIM13, LIM14, ELM04, ELM06, and ELM07).

Based on the *cytb* nucleotide sequences, 4 out of 12 isolates of *C. cassiicola* were found to have a mutation that replaces the codon 143 from GGT to GCT. This single mutation in the *cytb* gene of *C. cassiicola* isolates from soybean will change the translated protein from glycine (GGT) to alanine (GCT) that confers resistance to QoI-fungicides. The

G143A mutation was not found on isolates of *C. cassiicola* from cotton plants (Figure 2). No other point mutations on *cytb*, such as F129L and G137R, were found in our isolates.

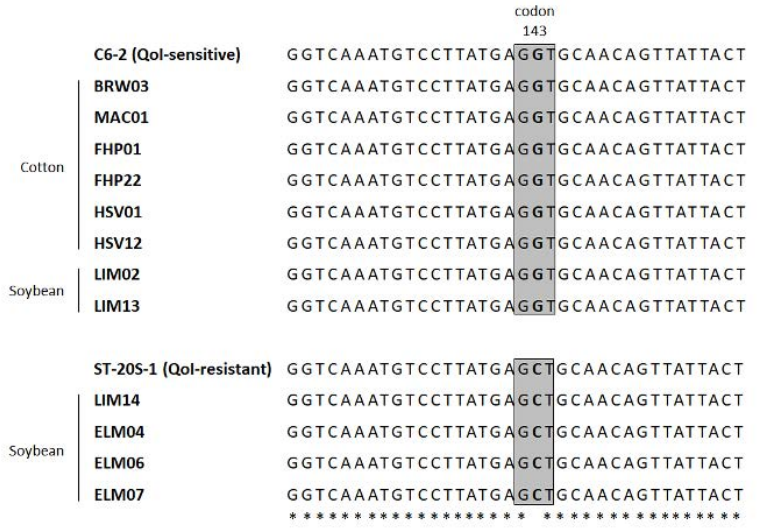

Figure 2. Partial nucleotide sequence of the *cytochrome b* gene of 12 isolates of *Corynespora cassiicola* plus two isolates used as reference, QoI-sensitive (C6-2) and QoI-resistant (ST-20S-1), from Ishii et al. (2007). Codon 143 is highlighted with the dark gray boxes, and the single nucleotide substitution is highlighted in bold (G**G**T → G**C**T).

The four *C. cassiicola* isolates identified with the G143A mutation were collected in north (LIM14) and central (ELM04, ELM06, ELM07) Alabama (Figure 3). This mutation is known to confer high levels of resistance to QoI fungicides, while low to moderate levels of resistance are the consequence of other mutations (F129L and G137R) (Duan et al. 2019).

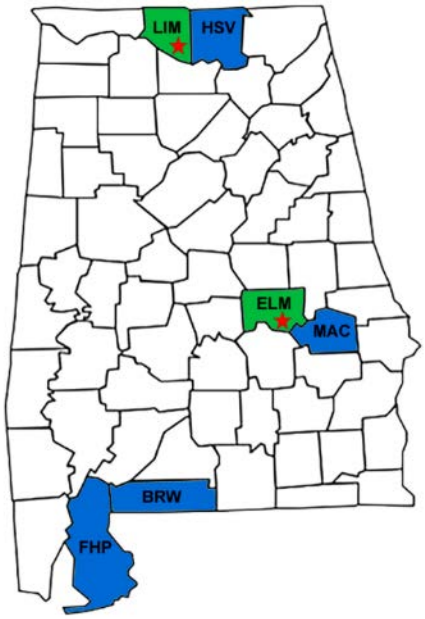

Figure 3. Distribution map of the *Corynespora cassiicola* isolates sampled from cotton (blue colored) and soybean (green colored) in Alabama, U.S. Abbreviations on the map represent the initial names of the isolates, and red stars those locations identified with the presence of G143A mutation on *C. cassiicola*.

According to FRAC (2019), the short development of resistance to different fungicide classes of *C. cassiicola* on soybean is one example of a pathogen that must be classified as a threatening. QoI fungicides have a single-site mode of action and are extensively applied to manage multiple diseases in field crops. Given the high-risk of *C. cassiicola* to develop fungicide resistance (Ishii et al. 2007, Duan et al. 2019), the management of fungicide resistance will be a major challenge. Knowing that field populations of *C. cassiicola* have mutations associated with QoI-resistance it will be necessary to monitor the spread of resistant isolates to manage resistance development. To the best of our knowledge, this study is the first to report G143A mutants in *C. cassiicola* from soybean field populations in the U.S.

Summary

Our results indicate an important discovery regardless of fungicide resistance. *Corynespora cassiicola* isolates collected from soybean were identified with a single nucleotide substitution in the *cytb* gene that results in an amino acid change from glycine to alanine (G143A). This mutation is known to confer high levels of resistance to QoI fungicides. *Corynespora cassiicola* isolates from cotton were not identified with G143A mutation. F129L and G137R are known to cause low to moderate levels of resistance to QoI fungicides but were not found in our samples. Our findings represent the first report of the occurrence of the G143A mutation associated with *C. cassiicola* in the U.S. These results will be helpful to monitor the presence of mutations in field populations of *C. cassiicola*, and to manage fungicide resistance.

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