

Title: Development of a regional fungicide resistance testing service for DMIs and QoIs using both conventional and molecular methods

Progress Report

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Objectives:

- 1) Optimization of methods for rapid fungicide testing
- 2) Provide fungicide testing service to growers and crop advisers

Justification and Description:

The University of Georgia Plant Pathology Department has recently established the Plant Molecular Diagnostic Laboratory (MDL) at the Tifton campus. This is a fee-based service lab, and the primary mission of the MDL is to provide advanced testing for plant pathogens via molecular and serological methods (PCR, RT-PCR, qPCR, LAMP, ELISA etc.) on plant samples submitted to the laboratory and affected by bacteria, fungi, viruses, or nematodes. However, fungicide resistance is a front-burner issue for growers and crop advisers over the last two to three decades. It can lead to lost disease control, reduced yields, and unnecessary expense by applying products that no longer work. There is no single location in the Southeast that can provide resistance testing for multiple fungal organisms and multiple fungicides. This proposal will start to address that need.

A limited number of fungicides are available to manage fungal pathogens, and this narrow fungicide pool increases the risk of disease control failure due to potential fungicide resistance development. The DMI and QoI fungicides have a specific mode of action towards a target protein in the fungal pathogens. A genetic adjustment by a fungus can lead to reduced sensitivity to these fungicides. The most important resistance mechanism is a modification of the fungicide target, caused by mutations in the encoding target gene and in some cases by overexpression of the target gene. For the proper management of fungal pathogens, we need to have early, rapid,

and accurate testing methods to identify fungicide resistance in various fungi. The overall goal of this proposed project is to optimize fungicide resistance testing for these fungicides at this lab and to establish a system to provide support to growers and crop advisers. This service will be crucial to guide growers to use effective fungicides to reduce losses caused by fungicide resistance. Though future testing will be on a fee basis, this initial funding will provide an opportunity for fungal organisms to be tested for resistance where putative field failures have occurred. Initial testing will be limited, but additional fungicide classes and fungi will be added over time.

Procedures:

A. Optimization of methods for rapid fungicide testing

- I. **Conventional plate tests:** Pure cultures of pathogens are isolated from infected plant samples and grown on agar plates (or other media), incubated for several days, then transferred to agar plates amended with a test fungicide at different concentrations. The plates will be incubated for 3 to 6 days (depending on the pathogen) to calculate fungicide efficacy. In general, this process can take more than 10 days before a diagnostic assessment can be made. In order to simplify and shorten this process, we will also develop a multi-well plate assay (Fig. 1). This assay allows testing of sensitivity of several fungal isolates to different fungicide concentrations on the same plate.

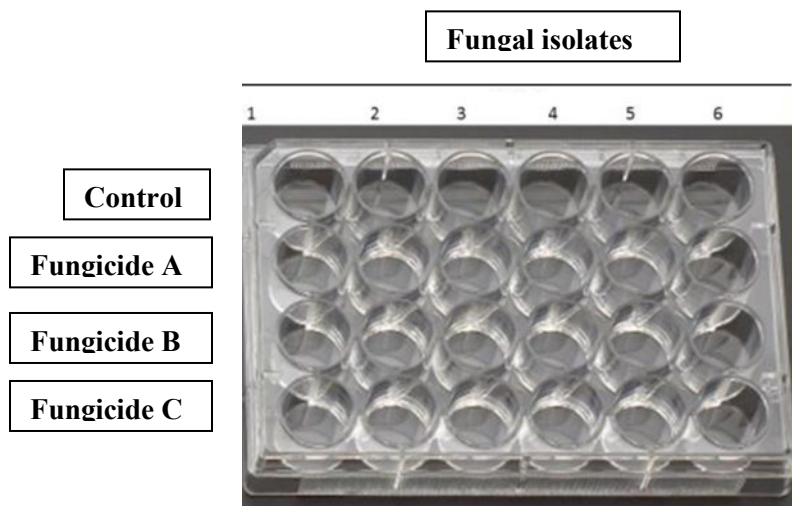


Figure 1. Multi-well plate assay (MWP) to evaluate the sensitivity of major fungicides

- II. **Molecular tests:** Using target site gene sequencing, pathogen resistance will be tested against respective fungicides based on previously published references. Fungal DNA will be extracted from infected samples using a Qiagen DNeasy Plant Mini Kit according to the manufacturer's instructions. Target site (DMI-Cytochrome P450 14 α -sterol demethylases, CYP51/QoI- mitochondrial cytochrome b gene, CYTB) will be amplified using polymerase chain reaction (PCR). PCR products will be sequenced and mutation(s) will be identified by comparing with NCBI sequence databases.

- B. Service:** Finally, fee-based testing services will be provided to small fruit crops extension & research personnel, commercial growers & homeowners, and various departments of agriculture.

Results and Current Progress

A. Optimization of methods for rapid fungicide testing

We have adapted the multi-well plate assay protocol from Dr. Guido Schnabel at Clemson University and transitioned their fungicide testing program to the molecular diagnostic laboratory at the University of Georgia. In brief for the MWP assay, suspected samples were incubated for several days (depending on pathogen's growth) in a moist chamber. This initial incubation process allows pathogen to grow and sporulate on sample surface. Then the pathogen was transferred onto the centers of fungicide-amended plates and nonamended control plates. Micro plates were incubated for 5 days at 22°C before measuring the radial growth in two perpendicular directions and determining pathogen's sensitivity to the respective fungicide. Three micro-plates were used for each fungicide and, sensitivity tests were repeated twice. In general, this MWP process take about ~7 days to take final decision on fungicide sensitivity. The molecular method was also optimized based on target site mutation in relation to fungicide resistant phenotype. It is widely reported that resistance to quinone outside inhibitors (QoIs) has been associated with the presence of amino acid substitution G143A in the cytochrome b gene (Ali et al., 2019). For further confirmation of the pyraclostrobin resistance phenotype, we have further examined the resistant isolates for the presence of the G143A substitution using the PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) assay as described Forcelini et al., 2018 (Figure 2).

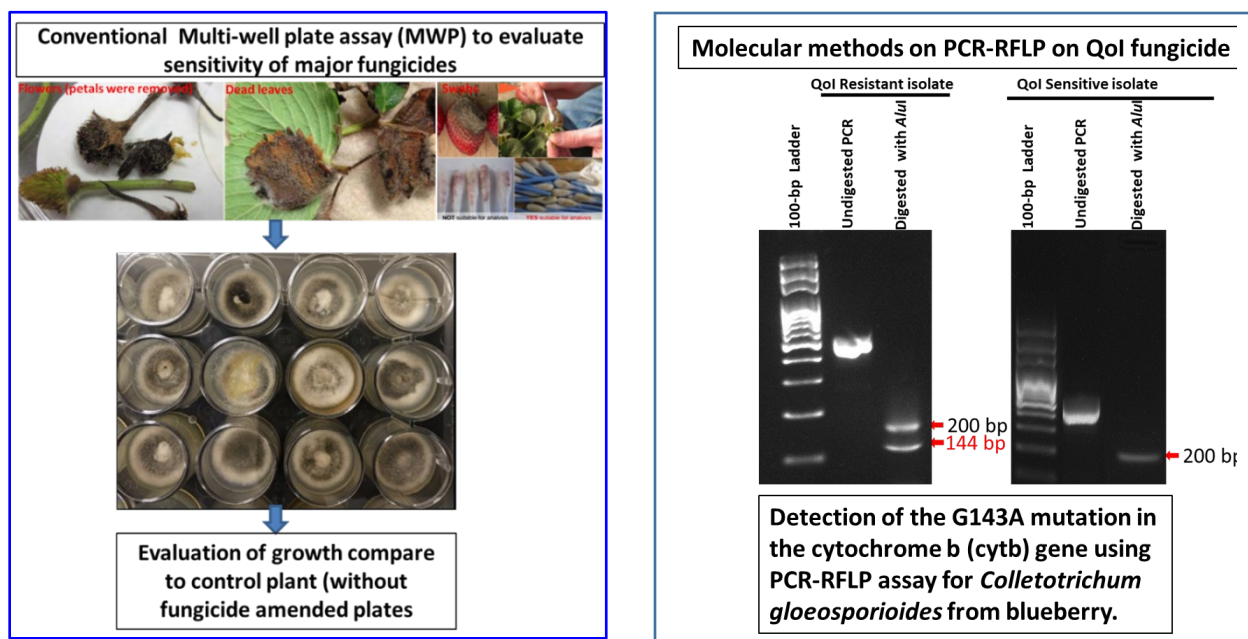


Figure 2: Optimization of methods for rapid fungicide testing

2) Provide fungicide testing service to growers and crop advisers

The MDL provided fungicide resistance testing support for *Colletotrichum* and *Botrytis* isolates received from small fruit growers and crop advisers. During 2019, MDL has received a total of 108 *Colletotrichum* isolates from 7 different farms and QoI (pyraclostrobin) sensitivity was tested via molecular and serological methods. A total of 60 *Botrytis* isolates were received from 6 farms and resistant profiling was done against 9 different chemistries. The overall results are shown below in Figures 3A and 3B. We are willing to conduct additional testing in 2020 for small fruit producers if the SRSFC provides an additional installment of funds.

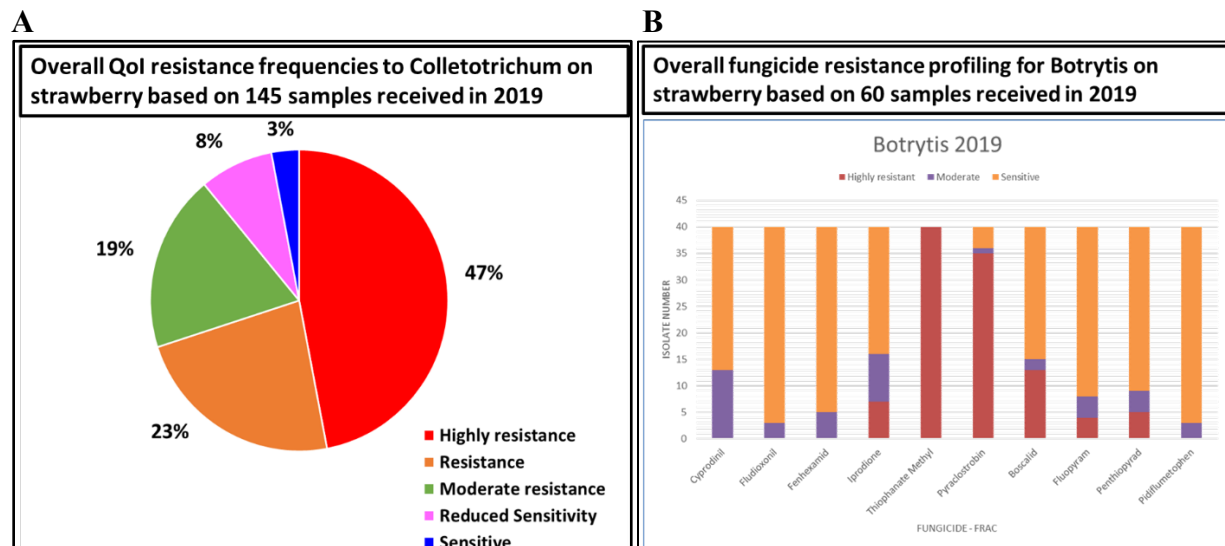


Figure 3: Fungicide resistance frequencies in 2019. A) QoI resistance frequencies for *Colletotrichum* isolates collected from 7 farms to QoI; B) Fungicide resistance frequencies for *Botrytis* isolates collected from 6 farms to different classes of fungicides.

Potential Impact

This service will be crucial to guide growers in the use of effective fungicides to reduce losses caused by fungicide resistance. Continuation of this testing service may lead to new management recommendations that will be valuable for small fruit growers in the southeastern U.S.

Literature Cited

1. Ali ME, Hudson O, Hemphill WH, Brenneman TB, Oliver JE. First Report of Resistance to Pyraclostrobin, Boscalid, and Thiophanate-methyl in *Colletotrichum gloeosporioides* from Blueberry in Georgia.0:261-262.
2. Forcelini, B.B., Rebello, C.S., Wang, N.Y., Peres, N.A. 2018. Fitness, Competitive Ability, and Mutation Stability of Isolates of *Colletotrichum acutatum* from Strawberry Resistant to QoI Fungicides. *Phytopathology* 108(4):462-468.