**PCR-based fungicide resistance screening in *Cercospora beticola* populations in Michigan, 2021-22**

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**Background:**

There are multiple fungicide groups that are commonly used and registered for Cercospora leaf spot (CLS) management in sugar beet including methyl benzimidazole carbamates (MBC or benzimidazole, FRAC group 1), quinone outside inhibitors (QoI or strobilurins, FRAC group 11), demethylation inhibitors (DMI or triazoles, FRAC group 3), organo-tins (FRAC group 30), and multi-site contact activity (FRAC group M03) fungicide classes. Reduced sensitivity to QoI, MBC, DMI, and organo-tin fungicides has been detected in *C. beticola* populations in Michigan (Weiland and Halloin 2001, Kirk et al. 2012, Bolton et al. 2012a, Rosenzweig et al. 2015, Rosenzweig et al. 2020). Because of the fluctuating levels of resistant isolates, continuous monitoring is necessary for prompt identification and proactive management of shifts in *C. beticola* sensitivities. PCR-based methods to detect mutations associated with fungicide resistance could provide timely and field specific guidance to improve CLS management, but they must provide information that is reliable and relevant to field efficacy of the compounds.

**Methods:**

CLS-symptomatic leaf samples were collected from mid-July through the end of October. Twenty-nine and thirty field locations were sampled in 2021 and 2022, respectively, across nine counties in east-central Michigan. Approximately eight lesions from 8-15 leaves were collected at each timepoint from each field site and mono-conidial isolates were obtained from each lesion.

Testing was conducted using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assays to detect point mutations in the *C. beticola* genome associated with fungicide resistance. QoI resistance was determined using the G143A point mutation present in the fungal mitochondrial cytochrome b gene of *C. beticola* isolates previously characterized to be resistant to pyraclostrobin, with EC50 values >100 ppm (Rosenzweig et al. 2015). MBC resistance was determined using the E198A point mutation present in the beta-tubulin gene of *C. beticola* isolates previously characterized to be resistant to benzimidazole, with EC50 values ≥ 60 ppm (Rosenzweig et al. 2015). DMI resistance was associated with the Glu169 (GAA to GAG) mutation present in the C-14 alpha-demethylase gene of *C. beticola* isolates characterized to be highly resistant to epoxiconazole, with EC50 values of 65-115 ppm (Nikou et al. 2009).

These rapid PCR-RFLP techniques were compared to current *in vitro* fungicide sensitivity testing methods. The effective concentrations required to inhibit mycelial growth by 50% (EC50) were determined through spiral gradient plating with each active ingredient of interest (Förster et al. 2004; Torres-Londoño et al. 2016; Rosenzweig et al. 2020). Isolates were tested for sensitivity to the QoI pyraclostrobin, the MBC thiophanate-methyl, the DMIs difenoconazole, tetraconazole, prothioconazole, fenbuconazole, and mefentrifluconazole, and the organotin, triphenyltin hydroxide.

**Results:**

*Objective 1* **-** Evaluate rapid testing as a tool to monitor *C. beticola* sensitivity to critical fungicide groups.

Results for the three PCR-RFLP assays were successfully obtained from 399 isolates in 2021 and 498 isolates in 2022. Of these, 63 isolates collected in 2021 were tested for in vitro fungicide sensitivity and compared with the PCR-RFLP results. The benzimidazole PCR marker predicted resistance to thiophanate-methyl with 100% accuracy. All the tested isolates contained the genetic mutation associated with QoI resistance. However, the pyraclostrobin EC50 values measured by spiral plating ranged from

0.79 ppm (lower limit of assay) to 88.37 ppm (upper limit). Resistance to triazoles is a complex trait controlled by multiple genes (Rangel et al. 2020). The mutation used in this study successfully predicted levels of insensitivity (> 1 µg/ml; Bolton et al. 2012b) for certain triazole fungicides (difenoconazole; Figure 1A) but not for others (tetraconazole; Figure 1B). This study will continue to explore other mutations associated with DMI resistance to tetraconazole (Spanner et al. 2021) and evaluate the mutations' ability to predict fungicide sensitivity.

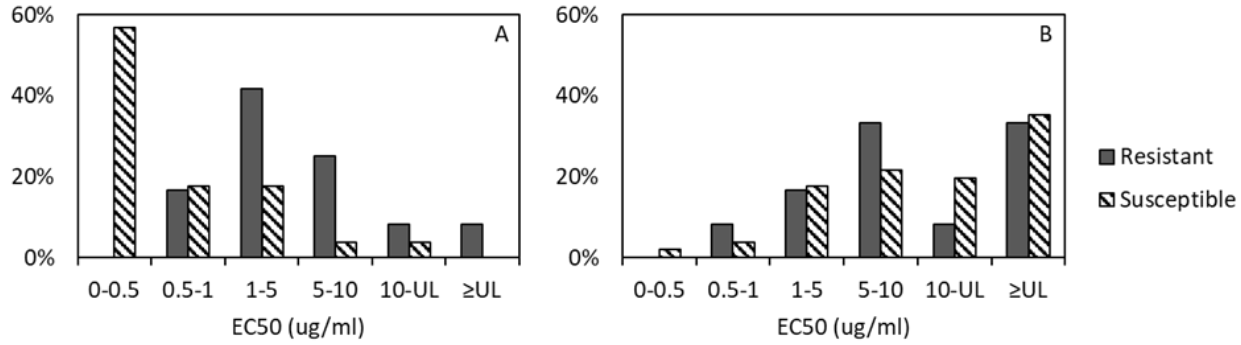


Figure 1. Isolate frequency distribution of *in vitro* fungicide sensitivity to (A) difenoconazole and (B) tetraconazole for *C. beticola* containing the mutation associated with high resistance (Resistant, N = 12; gray bars) and absence of the mutation meaning moderate resistant/susceptible (Susceptible, N = 51; striped bars) isolates (Nikou et al. 2009). The upper limit (UL) was 17.6 ppm for difenoconazole and 17.7 ppm for tetraconazole.

<u>*Objective 2*</u> - Monitor levels of resistance to critical fungicide groups across Michigan growing regions.

Some isolates with reduced sensitivity were identified for every active ingredient tested. Resistance to DMI fungicides varied by active ingredient; isolates of *C. beticola* exhibited the highest level of resistance to prothioconazole, followed by tetraconazole (Figure 2). High frequencies of resistance to pyraclostrobin were observed across Michigan (Figure 3). Some reduced sensitivity to triphenyltin hydroxide was observed for isolates tested in this study. However, the degree of resistance was lower than that of other fungicide classes with no isolates having EC50 values >10ppm (Figure 3). Resistance to low doses of organotin fungicides is being observed in North Dakota and Minnesota as well (Secor et al. 2019). Tables 1&2 show the percentage of isolates with reduced sensitivity for each of the field locations sampled. These frequencies are associated with *in vitro* EC50 values > 1 µg/ml active ingredient (Secor et al. 2010, Bolton et al. 2012b). While these values do not correspond directly to field-level resistance, regions with high frequencies of resistant isolates may be more likely to experience reduced efficacies with corresponding fungicide groups.

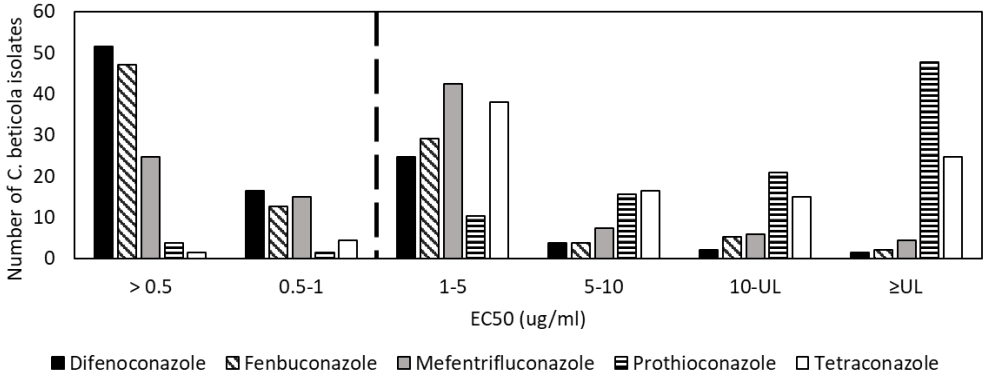


Figure 2. Isolate frequency distribution of in vitro fungicide sensitivity to difenoconazole (black), fenbuconazole (diagonal stripes), mefentrifluconazole (gray), prothioconazole (horizontal stripes), and tetraconazole (white) for *C. beticola* isolates. The

dashed line represents a resistance threshold of 1 ppm (Bolton et al. 2012b). All isolates to the right of the dashed line are considered to have some resistance. The upper limit (UL) was 17.6 ppm for difenoconazole, 17.9 ppm for fenbuconazole, 17.6 ppm for mefentrifluconazole, 17.8 ppm for prothioconazole, and 17.7 ppm for tetraconazole.

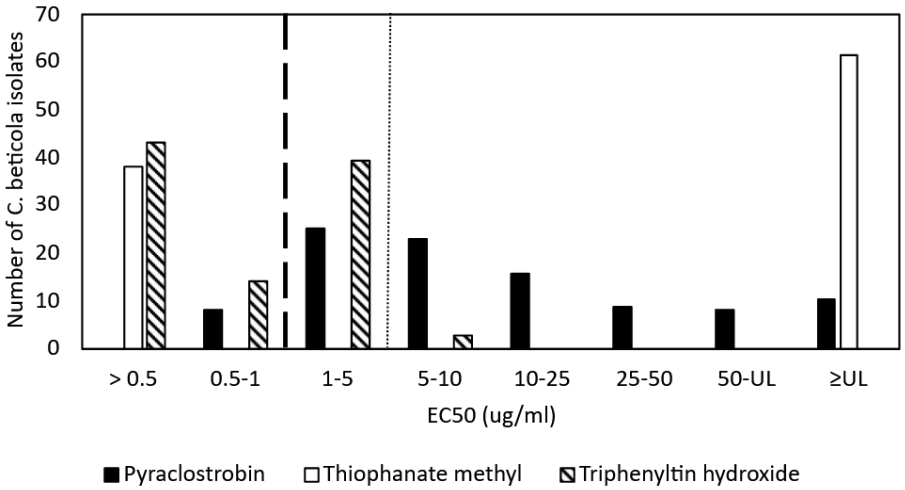


Figure 3. Isolate frequency distribution of *in vitro* fungicide sensitivity to a QoI, pyraclostrobin (black), an MBC, thiophanate methyl (white), and an organo-tin, triphenyltin hydroxide (diagonal stripes) for *C. beticola*. The dashed line represents a resistance threshold of 1 ppm used for pyraclostrobin and triphenyltin hydroxide. The dotted line represents a resistance threshold of 5 ppm used for thiophanate methyl (Secor et al. 2010). All isolates to the right of the corresponding threshold are considered resistant. The upper limit (UL) was 88.4 ppm for pyraclostrobin, 89.3 ppm for thiophanate methyl, and 17.8 ppm triphenyltin hydroxide.

**Summary**

- The PCR-RFLP rapid detection technique was accurate at predicting MBC resistance and can be deployed for screening isolates in future years. However, the genetic tests used in this study were not sufficient for accurately predicting QoI or DMI *in vitro* sensitivity for *C. beticola* isolates.
- Reduced sensitivity was observed for all active ingredients tested, but resistance was particularly widespread for the DMIs prothioconazole and tetraconazole as well as the QoI pyraclostrobin.

**Future Directions**

Isolates collected in 2022 will be tested using the spiral gradient method and compared to 2021 resistance levels to assess shifts in *C. beticola* populations. A subset of fields were sampled multiple times over the growing season and seasonal changes in resistance will be tracked and compared to the fungicide programs used. Fungicide sensitivities for *Alternaria alternata* isolates collected from similar Michigan sugar beet field locations will also be determined.

Additional mutations associated with DMI resistance will be tested for their ability to predict isolate sensitivity. Newer qPCR techniques (Shrestha et al. 2020) will also be investigated for rapid screening optimization. Collection and screening of symptomatic leaf samples will be repeated in 2023.

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**Table 1. Frequencies of *C. beticola* resistance to five triazole active ingredients detected using in vitro sensitivity testing in 2021**

| Date | Field Location | County | No. Samples | % Resistant[a] | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Difeno-conazole | Fenbu-conazole | Mefentriflu-conazole | Prothio-conazole | Tetra-conazole |
| 14-Jul | Munger | Bay | 4 | 50.0 | 0.0 | 75.0 | 100.0 | 100.0 |
| 15-Jul | Auburn | Bay | 4 | 25.0 | 0.0 | 75.0 | 100.0 | 100.0 |
| 15-Jul | Auburn | Bay | 4 | 25.0 | 0.0 | 75.0 | 100.0 | 100.0 |
| 22-Jul | Brown City | Sanilac | 3 | 66.7 | 0.0 | 66.7 | 66.7 | 100.0 |
| 27-Jul | Ashley | Gratiot | 5 | 0.0 | 0.0 | 60.0 | 80.0 | 80.0 |
| 16-Aug | Auburn | Bay | 3 | 66.7 | 33.3 | 100.0 | 100.0 | 100.0 |
| 16-Aug | Freeland | Saginaw | 3 | 33.3 | 33.3 | 0.0 | 100.0 | 100.0 |
| 17-Aug | Caseville | Huron | 4 | 0.0 | 50.0 | 25.0 | 100.0 | 100.0 |
| 25-Aug | Akron | Tuscola | 3 | 0.0 | 100.0 | 0.0 | 100.0 | 100.0 |
| 25-Aug | Gilford | Tuscola | 5 | 0.0 | 80.0 | 40.0 | 100.0 | 100.0 |
| 1-Sep | Ruth | Huron | 4 | 75.0 | 0.0 | 100.0 | 100.0 | 100.0 |
| 1-Sep | Freeland | Saginaw | 5 | 20.0 | 40.0 | 40.0 | 100.0 | 100.0 |
| 7-Sep | Crump | Bay | 6 | 50.0 | 50.0 | 50.0 | 100.0 | 100.0 |
| 7-Sep | Cass City | Tuscola | 5 | 40.0 | 80.0 | 40.0 | 100.0 | 100.0 |
| 13-Sep | Gladwin | Gladwin | 5 | 60.0 | 20.0 | 80.0 | 100.0 | 100.0 |
| 15-Sep | Midland | Midland | 5 | 20.0 | 20.0 | 40.0 | 80.0 | 100.0 |
| 16-Sep | Standish | Arenac | 4 | 50.0 | 25.0 | 100.0 | 100.0 | 100.0 |
| 16-Sep | Auburn | Bay | 5 | 60.0 | 60.0 | 100.0 | 100.0 | 100.0 |
| 17-Sep | Au Gres | Arenac | 3 | 33.3 | 33.3 | 66.7 | 100.0 | 100.0 |
| 17-Sep | Pinconning | Bay | 3 | 0.0 | 0.0 | 33.3 | 33.3 | 33.3 |
| 18-Sep | Brown City | Sanilac | 4 | 50.0 | 50.0 | 100.0 | 100.0 | 75.0 |
| 18-Sep | Croswell | Sanilac | 3 | 0.0 | 66.7 | 0.0 | 66.7 | 66.7 |
| 22-Sep | Freeland/Saginaw | Saginaw | 4 | 50.0 | 50.0 | 50.0 | 100.0 | 100.0 |
| 24-Sep | Beaverton | Gladwin | 5 | 80.0 | 60.0 | 100.0 | 100.0 | 100.0 |
| 3-Oct | Munger | Bay | 4 | 0.0 | 0.0 | 75.0 | 100.0 | 100.0 |
| 18-Oct | Sandusky | Sanilac | 5 | 0.0 | 100.0 | 20.0 | 100.0 | 100.0 |
| 21-Oct | Freeland | Saginaw | 5 | 40.0 | 60.0 | 40.0 | 80.0 | 80.0 |
| 23-Oct | Caseville | Huron | 6 | 33.3 | 50.0 | 66.7 | 100.0 | 83.3 |
| 24-Oct | Breckenridge | Gratiot | 5 | 40.0 | 60.0 | 40.0 | 80.0 | 80.0 |
| **Total** | **29 Locations** | **9 Counties** | **124** | **33.4** | **38.7** | **57.2** | **92.6** | **93.0** |

[a]Isolates with EC50 values ≥1µg/ml were considered resistant (Bolton et al. 2012b). While regions with high frequencies of resistant isolates are at greater risk for reduced efficacy of fungicides with these active ingredients, resistance rates are based on laboratory testing only and are not a direct measure of in-field control provided by these products.


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**Table 2. Frequencies of *C. beticola* resistance to QoI, MBC and organotin active ingredients detected using in vitro sensitivity testing in 2021**

| Date | Field Location | County | No. Samples | % Resistant[a] | | |
|---|---|---|---|---|---|---|
| | | | | Pyraclostrobin | Thiophanate methyl | Triphenyltin hydroxide |
| 14-Jul | Munger | Bay | 4 | 100.0 | 0.0 | 0.0 |
| 15-Jul | Auburn | Bay | 4 | 50.0 | 50.0 | 25.0 |
| 15-Jul | Auburn | Bay | 4 | 50.0 | 75.0 | 0.0 |
| 22-Jul | Brown City | Sanilac | 3 | 100.0 | 0.0 | 0.0 |
| 27-Jul | Ashley | Gratiot | 5 | 100.0 | 20.0 | 0.0 |
| 16-Aug | Auburn | Bay | 3 | 100.0 | 0.0 | 33.3 |
| 16-Aug | Freeland | Saginaw | 3 | 66.7 | 66.7 | 0.0 |
| 17-Aug | Caseville | Huron | 4 | 75.0 | 100.0 | 75.0 |
| 25-Aug | Akron | Tuscola | 3 | 100.0 | 100.0 | 33.3 |
| 25-Aug | Gilford | Tuscola | 5 | 80.0 | 100.0 | 20.0 |
| 1-Sep | Ruth | Huron | 4 | 100.0 | 50.0 | 50.0 |
| 1-Sep | Freeland | Saginaw | 5 | 100.0 | 80.0 | 80.0 |
| 7-Sep | Crump | Bay | 6 | 100.0 | 100.0 | 83.3 |
| 7-Sep | Cass City | Tuscola | 5 | 100.0 | 60.0 | 40.0 |
| 13-Sep | Gladwin | Gladwin | 5 | 100.0 | 60.0 | 60.0 |
| 15-Sep | Midland | Midland | 5 | 100.0 | 60.0 | 40.0 |
| 16-Sep | Standish | Arenac | 4 | 75.0 | 100.0 | 0.0 |
| 16-Sep | Auburn | Bay | 5 | 100.0 | 80.0 | 80.0 |
| 17-Sep | Au Gres | Arenac | 3 | 100.0 | 33.3 | 0.0 |
| 17-Sep | Pinconning | Bay | 3 | 100.0 | 0.0 | 100.0 |
| 18-Sep | Brown City | Sanilac | 4 | 100.0 | 25.0 | 25.0 |
| 18-Sep | Croswell | Sanilac | 3 | 100.0 | 66.7 | 66.7 |
| 22-Sep | Freeland/Saginaw | Saginaw | 4 | 75.0 | 75.0 | 25.0 |
| 24-Sep | Beaverton | Gladwin | 5 | 100.0 | 80.0 | 80.0 |
| 3-Oct | Munger | Bay | 4 | 100.0 | 100.0 | 0.0 |
| 18-Oct | Sandusky | Sanilac | 5 | 100.0 | 80.0 | 100.0 |
| 21-Oct | Freeland | Saginaw | 5 | 80.0 | 20.0 | 40.0 |
| 23-Oct | Caseville | Huron | 6 | 100.0 | 66.7 | 33.3 |
| 24-Oct | Breckenridge | Gratiot | 5 | 80.0 | 60.0 | 80.0 |
| **Total** | **29 Locations** | **9 Counties** | **124** | **90.7** | **58.9** | **40.3** |

[a]Isolates with EC50 values ≥1µg/ml for pyraclostrobin and triphenyltin hydroxide and ≥5µg/ml for thiophanate methyl were considered resistant (Secor et al. 2010, Bolton et al. 2012b). While regions with high frequencies of resistant isolates are at greater risk for reduced efficacy of fungicides with these active ingredients, resistance rates are based on laboratory testing only and are not a direct measure of in-field control provided by these products.