Fungicide resistance screening of *Cercospora beticola* populations in Michigan, 2022-23

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Background: Multiple fungicide groups 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) 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. In 2021, 2022, and 2023, east-central Michigan sugar beet fields were sampled across nine counties and 29, 30, and 17 locations, respectively. Approximately eight lesions from 8-15 leaves were collected at each timepoint and field site, and mono-conidial isolates were obtained from each lesion.

In vitro fungicide sensitivity testing was conducted using a spiral gradient plating method which determined the effective concentrations required to inhibit mycelial growth by 50% (EC_{50}) for each active ingredient of interest (Förster et al. 2004; Torres-Londoño et al. 2016; Rosenzweig et al. 2020). Isolates were tested for sensitivity to pyraclostrobin, thiophanate-methyl, difenoconazole, tetraconazole, prothioconazole, fenbuconazole (2021 only), mefentrifluconazole, and triphenyltin hydroxide.

In vitro methods were compared to rapid polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assays which detect point mutations 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 EC_{50} values $\geq$ 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 EC_{50} values of 65-115 ppm (Nikou et al. 2009).

Results:

<u>*Objective 1*</u> *- Monitor levels of resistance to critical fungicide groups across Michigan growing regions.* 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 1). Mefentrifluconazole and difenoconazole results were significantly positively correlated, indicating that the mechanisms of resistance may be similar between these two active ingredients (Figure 2). High frequencies of resistance to pyraclostrobin were observed across Michigan (Figure 1). 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 EC_{50} values >10 ppm. Resistance to low doses of organotin fungicides has also been observed in North Dakota and Minnesota (Secor et al. 2019).

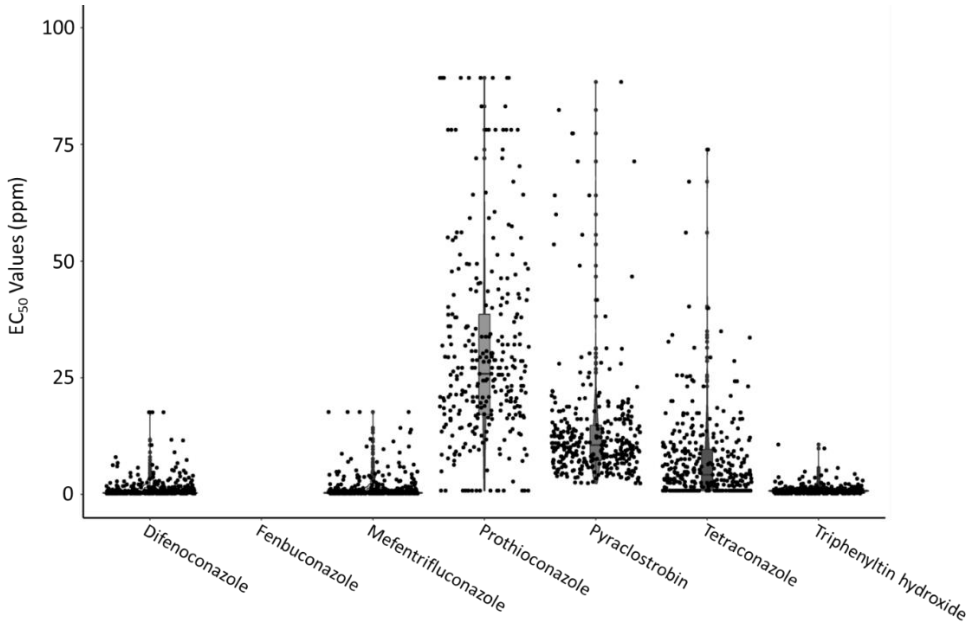

Figure 1. Box plots demonstrating the distribution of *in vitro* fungicide sensitivity to difenoconazole, fenbuconazole, mefentrifluconazole, prothioconazole, pyraclostrobin, tetraconazole, and triphenyltin hydroxide for *C. beticola* isolates collected in 2022. The upper limit of these assays were 17.6 ppm for difenoconazole, 17.9 ppm for fenbuconazole, 17.6 ppm for mefentrifluconazole, 17.8 ppm for prothioconazole, and 17.7 ppm for tetraconazole. The upper limit was 88.4 ppm for pyraclostrobin, 89.3 ppm for thiophanate methyl, and 17.8 ppm triphenyltin hydroxide.

Table 1. Frequencies of *C. beticola* resistance to four triazole active ingredients detected using *in vitro* sensitivity testing in 2022

County	No. locations	No. Samples	% Resistant[a]			
			Difenoconazole	Mefentrifluconazole	Tetraconazole	Prothioconazole
Arenac	3	12	50.0	16.7	33.3	66.7
Bay	22	124	35.8	38.2	70.5	92.3
Clinton	3	18	41.2	42.9	50.0	100.0
Gratiot	4	24	40.0	25.0	57.1	90.9
Huron	11	77	15.0	21.7	90.5	100.0
Midland	1	4	75.0	66.7	50.0	100.0
Saginaw	7	33	46.4	32.3	83.3	93.5
Sanilac	6	40	18.9	23.5	85.7	96.7
Tuscola	7	41	44.1	31.4	84.2	100.0
Total	**64**	**373**	**40.7**	**33.1**	**67.2**	**93.3**

[a] Isolates with EC_{50} values ≥1 ppm 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.

Table 2. Frequencies of *C. beticola* resistance to QoI, MBC and organotin active ingredients detected using *in vitro* sensitivity testing in 2022

			% Resistant[a]		
County	No. locations	No. Samples	Pyraclostrobin	Triphenyltin hydroxide	Thiophanate methyl
Arenac	3	12	100.0	10.0	-
Bay	22	124	100.0	25.2	91.7
Clinton	3	18	100.0	46.7	27.3
Gratiot	4	24	100.0	15.0	18.2
Huron	11	77	100.0	35.8	100.0
Midland	1	4	100.0	0.0	-
Saginaw	7	33	100.0	15.2	70.0
Sanilac	6	40	100.0	44.4	96.3
Tuscola	7	41	100.0	34.3	83.3
Total	**64**	**373**	**100.0**	**25.2**	**69.5**

[a]Isolates with EC_{50} values ≥1 ppm for pyraclostrobin and triphenyltin hydroxide and ≥5 ppm 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.

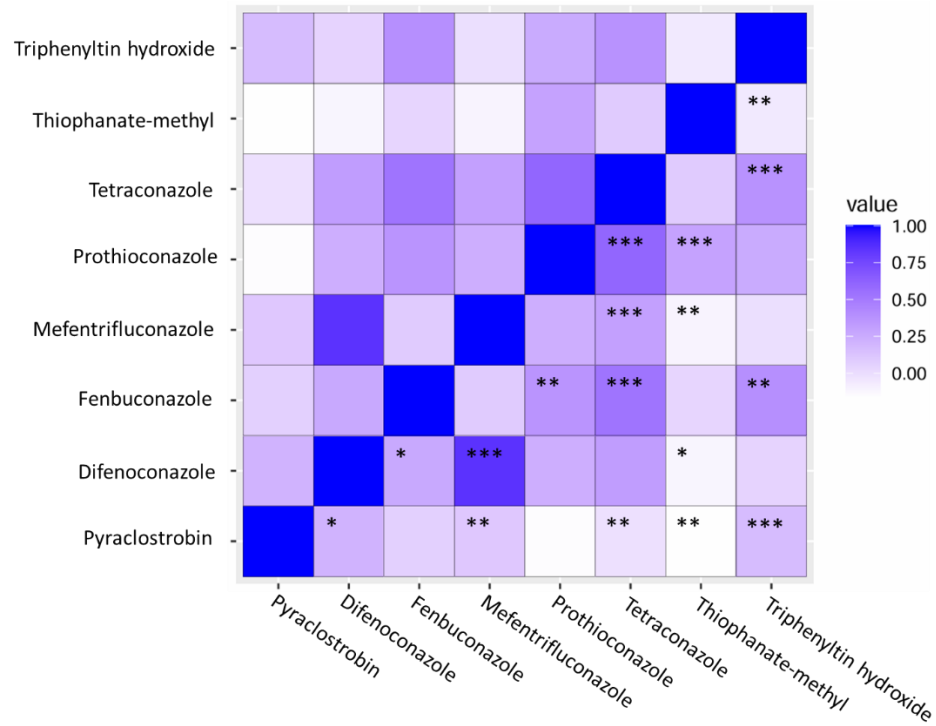

Figure 2. Heat map showing the correlation coefficients for *in vitro* fungicide sensitivity to difenoconazole, fenbuconazole, mefentrifluconazole, prothioconazole, pyraclostrobin, tetraconazole, and triphenyltin hydroxide for *C. beticola* isolates. Significance is denoted by the number of asterisks in the bottom right half of the heat map; a *p*-value < 0.05, < 0.01, and < 0.0001 is represented by '*', '**', and '***', respectively.

Objective 2 - 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. A subset of these (63 isolates in 2021 and 370 isolates in 2022) were tested for *in vitro* fungicide sensitivity and compared with the PCR-RFLP results. The benzimidazole PCR marker predicted resistance to thiophanate-methyl with 99% accuracy. In 2021, approximately 68% of isolates had the mutation associated with MBC resistance compared to 74% in 2022 while in 2022. All the tested isolates contained the genetic mutation associated with QoI resistance. However, the pyraclostrobin EC_{50} values measured by spiral plating ranged from 0.79 ppm (lower limit of assay) to 88.37 ppm (upper limit). *In vitro* sensitivity of *C. beticola* isolates was significantly impacted by the presence of the mutation associated with DMI resistance (p <0.0001). However, responses were not consistent between active ingredients. The mutation used in this study successfully predicted levels of insensitivity (> 1 ppm; Bolton et al. 2012b) for the triazoles difenoconazole and mefentrifluconazole but not for tetraconazole or prothioconazole. Resistance to triazoles is a complex trait controlled by multiple genes (Rangel et al. 2020). This study will continue to explore other mutations associated with DMI resistance to tetraconazole and prothioconazole (Spanner et al. 2021) and evaluate the mutations' ability to predict fungicide sensitivity.

Overall Summary:

- The PCR-RFLP rapid detection technique was highly accurate at predicting MBC resistance, and the number of *C. beticola* isolates with resistance is increasing from 2021 to 2022.
- The genetic tests used in this study were not sufficient for accurately predicting QoI or DMI *in vitro* sensitivity for *C. beticola* isolates.
- Insensitivity to active ingredient concentrations above 1 ppm 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 2023 will be tested using the spiral gradient method and compared to 2021 and 2022 resistance levels to assess shifts in *C. beticola* populations. A subset of fields was sampled multiple times over the growing season and seasonal changes in resistance will be tracked and compared to the fungicide programs used. 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.

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