Fungicide resistance screening for leaf spot pathogens of sugar beet, 2023-24

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Background: In 2015, Michigan growers reported increasing yield reduction caused by defoliation associated with Alternaria leaf spot (ALS) (Rosenzweig et al., 2017). Increased *in vitro* resistance has additionally been described for *Alternaria* spp. populations collected in Michigan fields (Rosenzweig et al., 2017; Rosenzweig et al., 2019). Interestingly, a potential biological trade-off for fungicide resistance has been proposed in the Cercospora leaf spot (CLS) pathosystem, as demethylation inhibitor (DMI) resistant isolates of *C. beticola* had increased sensitivities after being exposed to prolonged cold temperatures of -20°C (Karaoglanidis and Thanassoulopoulos, 2002; Arabiat et al., 2017). Studies of a potential biological trade-off in resistant *Alternaria* spp. isolates are currently lacking. Further research will guide management of beet leaf spot diseases in Michigan.

Objective 1: Characterize virulence and fungicide resistance of *Alternaria* spp. isolates from sugar beet. In 2022, 74 isolates of *Alternaria* spp. were collected across six Michigan counties and in 2023, 48 *Alternaria* isolates were collected. These isolates were then tested for virulence using a detached leaf-assay using 2-month-old sugar beets of the Alternaria leaf spot susceptible variety, CR-059. Spore suspensions were collected from pure isolate cultures and adjusted to $1x10^4$ conidia/ml using a hemocytometer. Lesion development was recorded daily beginning two days post inoculation for five days. This experiment was repeated twice.

Initial *in-vitro* fungicide sensitivities were collected for six fungicides active ingredients registered for management of leaf spot diseases in sugar beet in Michigan. These include four DMI fungicides (FRAC 3), difenoconazole, mefentrifluconazole, prothioconazole, and tetraconazole, as well as triphenyltin-hydroxide (FRAC 30), and thiophanate methyl (FRAC 1) (Rosenzweig et al., 2017; Rosenzweig et al., 2019). Plates were fungicide-amended using a gradient spiral dilution method (Förster et al, 2004) and spore suspensions were streaked onto them. The effective concentration to inhibit mycelial growth by 50% (EC_{50}) were determined four days post-inoculation. In the initial fungicide sensitivity screening, isolates were phenotypically categorized as previously defined by Rosenzweig et al. (2019) as resistant (EC_{50} >100 ppm), insensitive (EC_{50} = 50-100 ppm), moderately insensitive (EC_{50} = 10-50 ppm), reduced sensitive (EC_{50} = 1-10 ppm), and sensitive (EC50 <1 ppm) (Figure 1).

Results: In 2022, only 53% of the *Alternaria* spp. isolates caused significant lesion development ($P < 0.05$). Of the 74 isolates screened, 57 isolates resulted in more severe lesion development than the previously characterized P23 isolate. In 2023, 93% of the *Alternaria* isolates screened were significantly different from the control ($P < 0.05$). Water controls for both tests were negative for any lesion development. In both years of testing, the greatest frequencies of *in vitro* sensitivity (EC_{50} <1 ppm) were observed for difenoconazole and mefentrifluconazole (93-100% of *Alternaria* isolates) (Figure 1A and 1B). The greatest frequencies of *in vitro* insensitivity (EC_{50} 50-100+ ppm) were observed for thiophanate-methyl (81-100% of isolates). For triphenyltin hydroxide, most *Alternaria* isolates were categorized as sensitive to reduced sensitive (EC_{50} <1 to 10 ppm) (63-96%).

A

Active Ingredient	Sensitive (EC$_{50}$ <1 ppm)	Reduced Sensitive (EC$_{50}$ = 1-10 ppm)	Moderately Insensitive (EC$_{50}$ = 10-50 ppm)	Insensitive (EC$_{50}$ = 50-100 ppm)
Difenoconazole	93%	6%	-	-
Mefentrifluconazole	100%	-	-	-
Prothioconazole	27%	9%%	42%	22%
Tetraconazole	6%	40%	16%	37%
Thiophanate-methyl	-	7%	12%	81%
Pyraclostrobin	6%	10%	33%	51%
Triphenyltin hydroxide	2%	61%	20%	18%

B

Active Ingredient	Sensitive (EC$_{50}$ <1 ppm)	Reduced Sensitive (EC$_{50}$ = 1-10 ppm)	Moderately Insensitive (EC$_{50}$ = 10-50 ppm)	Insensitive (EC$_{50}$ = 50-100 ppm)
Difenoconazole	96%	4%	-	-
Tetraconazole	-	56%	33%	10%
Thiophanate-methyl	-	-	-	100%
Pyraclostrobin + SHAM	-	33%	10%	52%
Triphenyltin hydroxide	52%	46%	2%	-

Figure 1: Frequences for initial fungicide sensitivities across **A)** 74 *Alternaria* spp. isolates from 2022 and **B)** 48 *Alternaria* spp. isolates from 2023. The p-values denoting differences between isolates in 2022 were: 0.004 (mefentrifluconazole), 0.5 (difenoconazole), <0.0001 (prothioconazole), <0.001 (tetraconazole), <0.0001(thiophanate-methyl), and <0.001 (triphenyltin hydroxide). The p-values denoting differences between isolates in 2023 were: < 0.05 (difenoconazole), < 0.001 (tetraconazole), > 0.05 (thiophanate-methyl), and < 0.001 (triphenyltin hydroxide).

Objective 2: Evaluate potential cold temperature effects on fluctuations in fungicide sensitivity. Seven *Alternaria spp.* isolates (including the previously characterized *A. alternata* isolate P23 (Jayawardana, 2022)), and seven *C. beticola* from 2022 and 2023 were placed into three temperature-controlled environments (20, 4, and -20°C) using a split-plot design. Fungicide sensitivity was tested as previously described against difenoconazole, tetraconazole, thiophanate-methyl, or triphenyltin hydroxide. Screening began at two weeks and then continued every subsequent month for seven months.

Results: Preliminary data from the first year suggests that sensitivities of both *Alternaria* spp. and *C. beticola* isolates to triphenyltin-hydroxide were significantly impacted by time ($P < 0.05$) (Figure 2A and 2B). While the interaction between environment and time caused significant shifts in responses to difenoconazole ($P < 0.0001$) and tetraconazole ($P < 0.0001$) for *Alternaria* spp. isolates, no consistent shifts in resistance categories were observed. Environment did not induce significant changes to fungicide sensitivities for any of the active ingredients against *C. beticola*. Again, no other consistent shifts in resistance categories were observed for *C. beticola*. Data collection for the remaining timepoints of the second repetition of the experiment is in progress for *Alternaria* spp. and *C. beticola*.

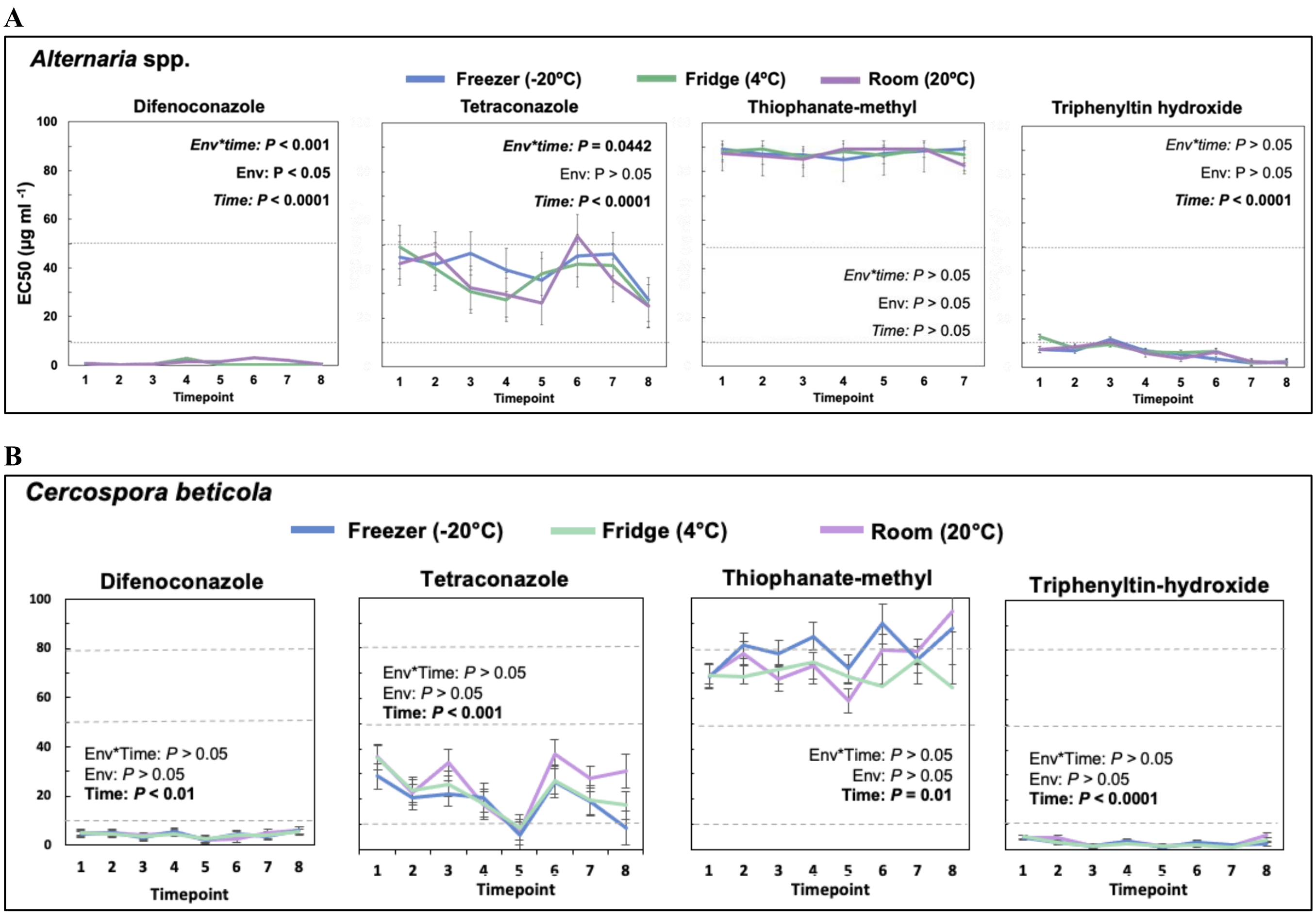

Figure 2: First year of mean EC_{50} values across *Alternaria* spp. and *C. beticola* isolates after incubation at 20°C, 4°C, or -20°C for up to seven months. Significance is denoted by p-value, with statistically significant results bolded.

Overall Summary:

- Similar levels of insensitivity were observed for tetraconazole and prothioconazole across *Alternaria* spp. isolates. Difenoconazole and mefentrifluconazole also had comparable responses with many isolates being classified as sensitive or reduced sensitive.
- A consistent shift to increased sensitivity was observed for triphenyltin-hydroxide, especially for *Alternaria* spp. isolates. The biological relevancy of these shifts could be investigated.

Future Directions: Data collection is ongoing for the cold-environment experiments. Additionally, virulence of *Alternaria* spp. isolates will be further characterized using a selection of commercial sugar beet varieties. The thresholds for sensitivity categories are also being reevaluated to identify a more biologically-relevant grouping system to better reflect resistance responses in these populations.

Acknowledgements: We thank the Michigan sugar beet industry for access to field sites and thank Michigan Sugarbeet Advancement and the Michigan Sugar Company for collection of sample materials. This work is supported by the Michigan Sugar Company, Michigan State University Project GREEEN, and USDA-ARS.