Fungicide Sensitivity Monitoring of *Alternaria* spp. Causing Leaf Spot of Sugarbeet (*Beta vulgaris*) in the Upper Great Lakes

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Abstract

Alternaria leaf spot (ALS), caused by *Alternaria* spp., can occur wherever sugarbeet is grown. Infection by *Alternaria* spp. and disease management has historically been considered a minor issue in sugarbeet production in the United States. An increase of both incidence and severity in 2016 of ALS high enough to cause yield loss has been observed in Michigan. With a renewed need to consider potential management of this disease, the sensitivity was determined for populations of *Alternaria* spp. to three classes of fungicides currently labeled for management of leaf spot on sugarbeet, including demethylase inhibitor (DMI), quinone outside inhibitor (QoI), and organo-tin fungicides. Leaves with symptoms of ALS were sampled from sugarbeet fields in east-central Michigan and southwestern Ontario, Canada. Monoconidial isolates were obtained to determine sensitivity to each fungicide class above. A spiral gradient

dilution method was used to estimate the fungicide effective concentration (in milligrams per liter) that caused a 50% inhibition of fungal growth in vitro for all isolates. Significant temporal shifts were detected in the frequencies of sensitivity phenotypes to DMI and QoI but not organo-tin fungicides from 2016 through 2017. Individual isolates of *Alternaria* spp. were recovered with cross-resistance to DMI and multiple resistance to DMI, QoI, and triphenyltin hydroxide fungicides. To our knowledge, this is the first report of a fungus other than *Cercospora beticola* with resistance to organo-tin fungicides. Fungicide sensitivity monitoring indicates that an effective integrated disease management approach combining fungicide efficacy trials and monitoring pathogen biology is essential for developing effective resistance management recommendations.

Alternaria leaf spot (ALS), caused by *Alternaria* spp. in the *A. alternata* and *A. brassicae* species groups, is an annually occurring disease wherever sugarbeet is grown, owing to the cosmopolitan nature of *Alternaria* spp. (Franc 2009). Infection by *Alternaria* spp. causes conspicuous necrotic spots on all aerial parts of the sugarbeet plant (Franc 2009). Subsequently, leaf spots expand and form lesions that may become uniform, concentric, and/or zonate (Franc 2009). Foliar infection by *Alternaria* spp. results in the reduction of photosynthetic area, accelerated senescence, and defoliation (Franc 2009). Infection by *Alternaria* spp. is commonly observed in association with *Beet western yellow virus* (BWYV) and other stresses that cause yellowing of the beet foliage (Franc 2009). ALS also can potentially affect sugar yield by increasing sodium content in the root (Russell 1965).

Infection by *Alternaria* spp. and disease management has historically been considered a minor issue in sugarbeet production in North America, owing to its opportunistic or secondary nature (Franc 2009; Russell 1965) and because damage from ALS usually does not significantly affect crop yield (Franc 2009). Additionally, the presence and severity of ALS has largely been attributed to a predisposition in

the sugarbeet crop as a result other biotic and abiotic stressors (Franc 2009). In contrast, ALS is a major concern in some sugarbeet production areas outside the United States, where the disease directly results in significant reductions in sugar yield (Abbas et al. 2014; Özgönen and Kılıç 2009). During the 2015 growing season, an increased incidence and severity of ALS was observed in Michigan sugarbeet production (Rosenzweig et al. 2017). Disease severity was observed at levels that previously were reported to have the potential to cause yield loss attributable to defoliation (Russell 1965). The increase in disease pressure of ALS was caused by conducive environmental conditions coupled with a significant percentage of sugarbeet production acreage planted with seed from ALS susceptible germplasm. Moreover, the levels of disease pressure in some growers' fields were high enough to raise potential concerns for sugarbeet production in the region. Little is known about the epidemiology of ALS related to *Cercospora* leaf spot (CLS) coinfection on sugarbeet. In the case of coinfection of sugarbeet by BWYV and ALS, an increase in susceptibility results from weakening of the whole plant (Russell 1965). The primary points of entry are yellow senescing leaves (Rotem 1994). *Alternaria* spp. have been shown to cause disease on both seed and seedlings of sugarbeet (Neergaard 1945). Additionally, *Alternaria* spp. can infect the inflorescence from which it passes to seeds and can lead to damping off of seedlings (Neergaard 1945). Because ALS has not been a concern in the United States, little is known about the interaction with varieties of beet host (Franc 2009) or sensitivity to currently available fungicides used in chemical management programs for foliar diseases. Conducive weather conditions for ALS are reported to include high humidity and low to moderate temperatures (Franc 2009). *A. brassicae* was reported to cause disease primarily at temperatures between 7 and 10°C, with no disease development above 16°C (McFarlane et al. 1954). Isolates in the *A. alternata* species group can be active at a wider temperature range but still have optimum development at cool temperatures, although specifics for beet are lacking. The optimum temperature for growth is around 25°C for *A. alternata* (syn. *A. tenuis*), with growth decreasing rapidly as temperature increases (Hasija 1970). ALS is most commonly seen in the Michigan growing region in early summer, decreases as summer temperatures rise, and increases again in the fall (*unpublished data*).

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Funding: This project was partially supported by the Michigan Sugar Company Research & Education Advisory Council and the Michigan State University Project GREEN (Generating Research and Extension to Meet Economic and Environmental Needs). Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the United States Department of Agriculture.

*The e-Xtra logo stands for “electronic extra” and indicates that four supplementary tables are published online.

The author(s) declare no conflict of interest.

Accepted for publication 30 March 2019.

Initial monitoring in 2016 of isolates of *Alternaria* spp. with high levels of insensitivity to many classes of fungicides were concomitantly recovered with *Cercospora beticola* causing CLS, the major foliar pathogen in the region from commercial production areas in Michigan and Ontario, Canada. Isolates of *Alternaria* spp. were tolerant to the quinone outside inhibitor (QoI), demethylase inhibitor (DMI), and triphenyltin hydroxide fungicides (Rosenzweig et al. 2017). Moreover, ALS is being increasingly observed in association with beets that also have lesions from *C. beticola* isolates tolerant to benzimidazole, QoI, and DMI fungicides (difenoconazole, tetraconazole, prothioconazole, and flutriafol). Resistance to these chemistries has been found in *C. beticola* in the region (Kirk et al. 2012; Rosenzweig et al. 2015; Trueman et al. 2013, 2017). Additionally, in 2015 and 2016, *Alternaria* spp. with insensitivity to the QoI fungicide pyraclostrobin (Fungicide Resistance Action Committee [FRAC] group 11) were recovered from fields in Michigan (Rosenzweig et al. 2017). Monoconidial isolates of *Alternaria* spp. recovered from individual ALS lesions were identified to species based on conidial morphology (Simmons 2007) and molecular-based methods (Woudenberg et al. 2013) by DNA sequencing (Rosenzweig et al. 2017). Sequence analysis revealed that intermediate and resistant isolates contained a substitution of G143A in the *cytB* gene (Rosenzweig et al. 2017), which confers QoI resistance in *A. alternata* (Grasso et al. 2006).

These results indicate the potential for the development of resistance in *Alternaria* isolates found in the region to currently used fungicides for leaf spot management. Management has been aimed at CLS but also may have indirectly impacted ALS in the past. Similar results have been reported in *Alternaria* spp. found on other crops with resistance to several different fungicide classes and the genetic mechanism is known (Avenot and Michailides 2007; Dewdney and Vega 2012; Ma and Michailides 2004a; Ma et al. 2003; Malandrakis et al. 2015). Typically, tolerance to QoIs and benzimidazole fungicides is attributable to mutations in the cytochrome *b* (Avila-Adame et al. 2003; Bartlett et al. 2002; Chin et al. 2001; Ishii et al. 2001; Küng Färber et al. 2002; Olaya et al. 2003; Pasche et al. 2005; Rosenzweig et al. 2008a, b, 2017) and β -tubulin genes (Cooley and Caten 1993; Davidse and Flach 1977; Jung et al. 1992; Reijo et al. 1994) respectively. These mutations cause codon substitutions and result in amino acid changes that reduce the binding specificity of the given fungicides at the target site.

Management problems related to application timing of fungicides and development of insensitivity to fungicides in pathogen populations causing leaf spot of sugarbeet (e.g., CLS) have impacted sugarbeet production regionally (Rosenzweig et al. 2015). The limited availability of effective active ingredients and significant economic cost necessitates the application of products with similar modes of actions in the same fungicide class and a reduction in total applications over the growing season to manage foliar diseases of sugarbeet in the Upper Great Lakes Region. Because of the potentially wide host range of *Alternaria* spp. causing ALS (Franc 2009), these pathogens have the potential to be exposed to these same fungicides even when sugarbeet is not present or planted. In addition, management can be hampered because some of the fungicides may not even be labeled for ALS. With the increasing incidence and potential for losses in the region with this pathogen, fungicide resistance monitoring in sugarbeet production areas in Michigan and Ontario is warranted.

Sensitivity monitoring is essential for the development of fungicide resistance management recommendations (Bauske et al. 2018; Thomas et al. 2012). Moreover, information on the population biology of plant pathogens related to fungicide sensitivity is important to evaluate the efficacy of available fungicides (Bauske et al. 2018) as well as the potential impact of management on long-term utility of various compounds (e.g., recurrence of tin sensitivity). The objective of this study was to establish a monitoring program by (i) collecting ALS-infected sugarbeet leaves from commercial production fields in eastern Michigan and western Ontario, Canada; (ii) determining the sensitivity of the recovered populations of *Alternaria* spp. to three classes of fungicides, including DMI fungicides (difenoconazole, fenbuconazole, flutriafol, prothioconazole, and tetraconazole), a QoI fungicide (pyraclostrobin), and an organo-tin (triphenyl tin

OH); and (iii) determining temporally the frequency of fungicide sensitivity phenotypes from 2016 through 2017 across the sugarbeet production area of the Upper Great Lakes.

Materials and Methods

Isolates and sampling. Isolates of *Alternaria* spp. were collected from leaves with symptoms of ALS sampled from sugarbeet fields in east-central Michigan during September and October in 2016 (60 fields) and 2017 (82 fields). Samples of up to 20 leaves from throughout a selection of sugarbeet production fields in Michigan and Ontario were used in sensitivity testing. Isolates of *Alternaria* spp. were recovered from infected leaf tissue from multiple field locations using standard methods (Rosenzweig et al. 2008a, b). Monoconidial isolates from individual ALS lesions were obtained to determine sensitivity to each fungicide described below. From each sugarbeet leaf, representative lesions were cut with a number 5 cork-borer; the lesions were chosen as being of similar sizes and in similar stages of sporulation and surface disinfested with 0.6% NaClO. All single lesion leaf disks derived from a particular sampling site were placed onto 1.5% water agar media amended with streptomycin (50 mg/liter) and CaCO₃ (0.15% wt/vol) for sporulation induction and positive identification. One conidium of *Alternaria* sp. from individual lesions was transferred to clarified V-8 (CV8) media (Miller 1955) amended with streptomycin and CaCO₃ for subsequent fungicide sensitivity assays and long-term storage.

In vitro fungicide sensitivity of *Alternaria* spp. by dilution gradient. Active ingredients of difenoconazole (Inspire; FRAC group 3), fenbuconazole (Enable; FRAC group 3), flutriafol (Topguard; FRAC group 3), prothioconazole (Proline; FRAC group 3), tetraconazole (Eminent125 SL; FRAC group 3), pyraclostrobin (Headline 2.08SC; FRAC group 11), and triphenyl tin OH (Super Tin 80WP; FRAC group 30) were prepared as stock solutions of 10,000 mg/liter of each fungicide by dissolving technical-grade fungicides in an appropriate sterile solvent (ethanol or acetone). Previous studies determined that salicylhydroxamic acid (SHAM) was toxic to germinating conidia for isolates tested; thus, the effective concentration in inhibiting growth by 50% (EC₅₀) values for pyraclostrobin were based on assays from fungicide-only amended agar (Rosenzweig et al. 2017). Subsequently, 50 ml of fungicide-amended and nonamended CV8 agar was poured into each dish, to form a layer of agar with a constant volume; thus when a stock solution is added to the agar, it results in a gradient from 0 to 1,000 mg/liter across the agar surface. The EC₅₀ value was determined for individual isolates. A spiral gradient plater method was used to determine the EC₅₀ value (Förster et al. 2004). The spiral gradient method was selected based on cost, efficiency, and relative accuracy. Conidial suspensions were prepared by flooding 2-week-old pure *Alternaria* spp. cultures grown on Petri dishes with 1 ml of distilled water and scraping the conidia free from the surface with a rubber policeman. An aliquot of the conidial suspension (10 μ l) was spread across the fungicide gradient plate from the edge to the center. Isolates were incubated for 7 days at 24°C in the dark (three replications). The point coordinates at which the colony growth began and ended was recorded and entered into the ECX package (Torres-Londoño et al. 2016) in the RStudio software program (Boston, MA), and the EC₅₀ value for each isolate and fungicide was calculated.

Data analysis. Categorical data were analyzed using a log-linear analysis approach based on collection year. The CATMOD procedure was employed using SAS software (Statistical Analysis System, version 9.3; SAS Institute) with log link. Additionally, PROC CATMOD provided maximum-likelihood estimates for the main effects of year.

Results

Determination of temporal shifts in fungicide sensitivity of *Alternaria* spp. The sensitivity of the recovered populations of *Alternaria* spp. was determined for five DMIs as well as one QoI and one organo-tin fungicide. A total of 143, 140, 131, 143, 144, 131, and 147 isolates of *Alternaria* spp. were screened against difenoconazole, fenbuconazole, flutriafol, prothioconazole, tetraconazole,

pyraclostrobin, and triphenyl tin OH fungicides, respectively, in 2016 (Table 1). A total of 236, 243, 250, 247, 240, 241, and 249 isolates of *Alternaria* spp. were screened against difenoconazole, fenbuconazole, flutriafol, prothioconazole, tetraconazole, pyraclostrobin, and triphenyl tin OH fungicides, respectively, in 2017 (Table 1).

For difenoconazole, the mean EC₅₀ values were 3.8 and 5.7 mg/liter in 2016 and 2017, respectively (Table 1). The mean EC₅₀ values ranged from 0.6 to 7.2 mg/liter among counties in 2016 (Supplementary Table S1) and from 0.7 to 12.9 mg/liter among counties and Ontario, respectively, in 2017 (Supplementary Table S2).

For fenbuconazole, the mean EC₅₀ values were 79.3 and 86.8 mg/liter in 2016 and 2017, respectively (Table 1). The mean EC₅₀ values ranged from 3.7 to 98.5 mg/liter among counties in 2016 and from 62.0 to 133.6 mg/liter among counties and Ontario, respectively, in 2017.

For flutriafol, the mean EC₅₀ values were 52.7 and 73.5 mg/liter in 2016 and 2017, respectively (Table 1). The mean EC₅₀ values ranged from 0.8 to 110.0 mg/liter among counties in 2016 and from 8.6 to 95.1 mg/liter among counties and Ontario, respectively, in 2017.

For prothioconazole, the mean EC₅₀ values were 71.7 and 53.5 mg/liter in 2016 and 2017, respectively (Table 1). The mean EC₅₀ values ranged from 0.7 to 106.3 mg/liter among counties in 2016 and from 24.9 to 86.1 mg/liter among counties and Ontario, respectively, in 2017.

For tetraconazole, the mean EC₅₀ values were 102.1 and 76.9 mg/liter in 2016 and 2017, respectively (Table 1). The mean EC₅₀ values ranged from 42.0 to 136.5 mg/liter among counties in 2016 and from 38.9 to 116.4 mg/liter among counties and Ontario, respectively, in 2017.

For pyraclostrobin, the mean EC₅₀ values were 59.0 and 91.7 mg/liter in 2016 and 2017, respectively (Table 1). The mean EC₅₀ values ranged from 0.6 to 128.0 mg/liter among counties in 2016 (Supplementary Table S3) and from 35.9 to 128.0 mg/liter among counties and Ontario, respectively, in 2017 (Supplementary Table S4).

For triphenyl tin OH, the mean EC₅₀ values were 16.5 and 39.0 mg/liter in 2016 and 2017, respectively (Table 1). The mean EC₅₀ values ranged from 0.7 to 63.3 mg/liter among counties in 2016 and from 33.1 to 52.6 mg/liter among counties and Ontario, respectively, in 2017.

Determination of temporal frequency distribution of fungicide sensitivity in *Alternaria* spp. For difenoconazole, the relative frequency of isolates with EC₅₀ values of <1, 1 to 5, 5 to 10, and 10 to 50 mg/liter was 50.3, 32.2, 11.9, and 5.6%, respectively, in 2016 (Fig. 1) compared with the relative frequency of isolates with EC₅₀ values in the same classes of 50.8, 33.9, 5.9, 5.5, 0.8, and 1.7% respectively, in 2017 (Fig. 1). The distribution of isolates in

these sensitivity classes to the other fungicides was determined over the 2 years of monitoring (Figs. 2, 3, 4, 5, 6, and 7). Of particular note are those for tetraconazole, which showed a different pattern in the relative frequency of isolates with EC₅₀ values of 1 to 5, 5 to 10, 10 to 50, 50 to 100, and >100 mg/liter of 1.4, 0.7, 22.9, 9.7, and 65.3% in 2016 and 0.4, 3.8, 7.9, 27.9, 17.1, and 42.5% in 2017, respectively (Fig. 5).

Temporal comparison of population resistance factors in *Alternaria* spp. The monitoring program for *Alternaria* spp. was initiated in the last three growing season and could not be characterized as baseline because of the pathogen's wide host range isolates (no exposure). Therefore, isolates characterized as sensitive were based on previously published data on mean sensitivity of *A. alternata* to the studied fungicides (Avenot et al. 2016; Dewdney and Vega 2012; Iacomini-Vasilescu et al. 2004). Isolates of *Alternaria* spp. were categorized phenotypically into five sensitivity categories based on mean EC₅₀ values as follows: EC₅₀ < 1 mg/liter, sensitive (S); EC₅₀ of 1 to 10 mg/liter, reduced sensitive (RS); EC₅₀ of 10 to 50 mg/liter, moderately insensitive (MI); EC₅₀ of 50 to 100 mg/liter, insensitive (I); and EC₅₀ > 100 mg/liter, resistant (R). The resistance factor (RF), based on EC₅₀ values, was calculated for populations of *Alternaria*

Fig. 1. Frequency distributions of in vitro sensitivity to difenoconazole of *Alternaria* spp. isolates recovered in Michigan from sugarbeet leaves collected from 2016 and 2017. Sensitivity expressed as 50% inhibition of fungal growth (EC₅₀) in vitro, fungicide concentration estimate determined by the spiral gradient dilution method.

Table 1. Comparison of mean effective concentration for reduction in growth by 50% (EC₅₀) for isolates of *Alternaria* spp. from sugarbeet in Michigan to seven fungicides in 2016 and 2017

Active ingredient	FRAC code ^a	Total no. of isolates	EC ₅₀ (mg/liter) ^b		
			Mean ± S.E.M.	Minimum	Maximum
2016					
Difenoconazole	3	143	3.8 ± 0.5	0.6	37.4
Fenbuconazole	3	140	79.3 ± 4.6	0.7	133.6
Flutriafol	3	131	52.7 ± 4.4	0.8	137.8
Prothioconazole	3	143	71.7 ± 4.8	0.7	132.7
Tetraconazole	3	144	102.1 ± 3.6	4.0	136.5
Pyraclostrobin	11	131	59.0 ± 4.4	0.6	128.0
Triphenyl tin OH	30	147	16.5 ± 0.9	0.7	63.3
2017					
Difenoconazole	3	236	5.7 ± 1.2	0.6	126.2
Fenbuconazole	3	243	86.8 ± 3.4	0.7	133.6
Flutriafol	3	250	73.5 ± 3.6	0.8	137.8
Prothioconazole	3	247	53.5 ± 3.5	0.7	132.7
Tetraconazole	3	240	76.9 ± 3.4	0.8	136.5
Pyraclostrobin	11	241	91.7 ± 3.3	0.6	128.0
Triphenyl tin OH	30	249	39.0 ± 1.5	0.7	130.2

^a FRAC = Fungicide Resistance Action Committee group name based on chemical relatedness and mode of action.

^b EC₅₀ values determined for two replications based on mean effective concentration for reduction in growth by 50% using a spiral gradient dilution method.

Fig. 2. Frequency distributions of in vitro sensitivity to fenbuconazole of *Alternaria* spp. isolates recovered in Michigan from sugarbeet leaves collected from 2016 and 2017. Sensitivity expressed as 50% inhibition of fungal growth (EC_{50}) in vitro, fungicide concentration estimate determined by the spiral gradient dilution method.

Fig. 5. Frequency distributions of in vitro sensitivity to tetraconazole of *Alternaria* spp. isolates recovered in Michigan from sugarbeet leaves collected from 2016 and 2017. Sensitivity expressed as 50% inhibition of fungal growth (EC_{50}) in vitro, fungicide concentration estimate determined by the spiral gradient dilution method.

Fig. 3. Frequency distributions of in vitro sensitivity to flutriafol of *Alternaria* spp. isolates recovered in Michigan from sugarbeet leaves collected from 2016 and 2017. Sensitivity expressed as 50% inhibition of fungal growth (EC_{50}) in vitro, fungicide concentration estimate determined by the spiral gradient dilution method.

Fig. 6. Frequency distributions of in vitro sensitivity to pyraclostrobin of *Alternaria* spp. isolates recovered in Michigan from sugarbeet leaves collected from 2016 and 2017. Sensitivity expressed as 50% inhibition of fungal growth (EC_{50}) in vitro, fungicide concentration estimate determined by the spiral gradient dilution method.

Fig. 4. Frequency distributions of in vitro sensitivity to prothioconazole of *Alternaria* spp. isolates recovered in Michigan from sugarbeet leaves collected from 2016 and 2017. Sensitivity expressed as 50% inhibition of fungal growth (EC_{50}) in vitro, fungicide concentration estimate determined by the spiral gradient dilution method.

Fig. 7. Frequency distributions of in vitro sensitivity to triphenyl tin OH of *Alternaria* spp. isolates recovered in Michigan from sugarbeet leaves collected from 2016-2017. Sensitivity expressed as 50% inhibition of fungal growth (EC_{50}) in vitro, fungicide concentration estimate determined by the spiral gradient dilution method.

spp. characterized as RS (EC_{50RS}/EC_{50S}), MI (EC_{50MI}/EC_{50S}), I (EC_{50I}/EC_{50S}), and R (EC_{50R}/EC_{50S}). The RF was calculated for populations of *Alternaria* spp. sensitive to all seven fungicides tested (Tables 2 and 3).

For difenoconazole, the RFs of populations of RS and MI were 6.3 and 35.3, respectively, in 2016 (Table 2), whereas the RFs of populations of RS, MI, I, and R were 4.8, 32.6, 105.4, and 143.4, respectively, in 2017. Similar patterns were seen for other DMI fungicides (Table 2).

For pyraclostrobin, the RFs of populations of RS, MI, I, and R were 8.8, 46.2, 108.4, and 202.9 in 2016 (Table 3). The RFs of populations of RS, MI, I, and R were 6.9, 37.9, 115.7, and 201.4, respectively, in 2017 (Table 3).

For triphenyl tin OH, the RFs of populations of RS, MI, and I were 12.4, 28.4, and 86.7 in 2016 (Table 3). The RFs of populations of RS, MI, I, and R were 10.1, 46.2, 98.6, and 185.7, respectively, in 2017 (Table 3).

Determining temporal frequency distribution of *Alternaria* spp. fungicide sensitivity phenotypes. For difenoconazole, log-linear analysis determined that collection year had a significant effect on the frequency of recovering *Alternaria* spp. S ($\chi^2 = 37.00$, $P < 0.0001$), RS ($\chi^2 = 15.92$, $P < 0.0001$), MI ($\chi^2 = 9.76$, $P = 0.0018$), and R ($\chi^2 = 15.37$, $P < 0.0001$) sensitivity phenotypes (Table 4). The probability of recovering *Alternaria* spp. isolates of S ($P < 0.0001$), RS ($P < 0.0001$), and R ($P < 0.0001$) phenotypes was significantly higher in 2017 than 2016 and was lower for MI phenotypes ($P = 0.0018$) over the same years (Table 5).

For fenbuconazole, log-linear analysis determined that collection year had a significant effect on the frequency of recovery of *Alternaria* spp. RS phenotypes ($\chi^2 = 45.86$, $P < 0.0001$) on the frequency of RS *Alternaria* spp. isolates collected (Table 4). The probability of collecting RS *Alternaria* spp. isolates was significantly higher in 2017 (Table 5) than 2016.

For flutriafol, log-linear analysis determined that collection year had a significant effect on the frequency of recovery of *Alternaria* spp. I ($\chi^2 = 15.11$, $P = 0.0001$) and R ($\chi^2 = 5.63$, $P = 0.0177$) sensitivity phenotypes (Table 4). The probability of recovering both I and R flutriafol phenotypes of *Alternaria* spp. isolates was significantly higher in 2017 than 2016 (Table 5).

For prothioconazole, log-linear analysis determined that collection year had a significant effect on the frequency of recovery of *Alternaria* spp. S ($\chi^2 = 8.86$, $P = 0.0029$) and RS ($\chi^2 = 7.20$, $P = 0.0073$) sensitivity phenotypes (Table 4). The probability of recovering *Alternaria* spp. isolates of S and RS prothioconazole phenotypes was significantly higher in 2017 than 2016 (Table 5).

For tetraconazole, log-linear analysis determined that collection year had a significant effect on the frequency of recovery of *Alternaria* spp. R ($\chi^2 = 4.35$, $P = 0.0371$) sensitivity phenotypes (Table 4). The probability of collecting R *Alternaria* spp. isolates was significantly lower in 2017 (Table 5).

For pyraclostrobin, log-linear analysis determined that collection year had a significant effect on the frequency of recovery of *Alternaria* spp. sensitivity R ($\chi^2 = 23.98$, $P < 0.0001$) phenotypes (Table 6). The probability of recovering R *Alternaria* spp. isolates was significantly higher in 2017 ($P < 0.0001$) (Table 6). For triphenyl tin OH, log-linear analysis determined that collection year did not have a significant effect on the frequencies of *Alternaria* spp. sensitivity phenotypes (Table 6).

Discussion

Alternaria spp. with high levels of insensitivity to three classes of fungicides were recovered from leaves showing leaf spot symptoms from commercial sugarbeet production areas in the Upper Great Lakes. Management of CLS on sugarbeet relies on an integrated approach combining cultural practices, resistant varieties, and application programs using a combination of protectant (e.g., dithiocarbamates and organo-tins) and systemic (e.g., DMIs and QoIs) fungicides (Secor et al. 2010b). Because CLS is the most important foliar disease of sugarbeet, management of ALS has historically been a minor issue and likely occurred as a consequence of foliar applications of fungicides for CLS. Therefore, the development of fungicide resistant populations of *Alternaria* spp. has emerged as a new problem and may be a factor, at least in part, in an increase of ALS in sugarbeet-producing areas in the Upper Great Lakes. This increased incidence and severity of ALS has been observed at levels high enough to potentially cause yield loss (Russell 1965). The current research represents a comprehensive study of the population biology related to fungicide sensitivity of the *Alternaria* species complex responsible for ALS on commercial sugarbeet production in the Upper Great Lakes. This study determined the sensitivity of the recovered populations of *Alternaria* spp. to seven different chemistries (difenoconazole, fenbuconazole, flutriafol, prothioconazole, tetraconazole, pyraclostrobin, and triphenyl tin OH) in the DMI, QoI and organo-tin classes of fungicide. Subsequently, isolates of *Alternaria* spp. were categorized phenotypically based on mean sensitivity into S, RS, MI, I, and R and the RF was calculated for these *Alternaria* spp. classes to the seven fungicides. Additionally, the current study determined significant temporal shifts in *Alternaria* spp. fungicide sensitivity phenotypes from 2016 compared with 2017.

For the DMI fungicides, difenoconazole had the lowest mean EC_{50} and variability of sensitivity in both sampling years. Among all fungicides tested, the mean EC_{50} increased from 2016 to 2017 except in the case of tetraconazole where the mean EC_{50} decreased. Because this monitoring program was initiated in the last three growing seasons and is presumably attributable to the wide host range of *Alternaria* spp. and persistent fungicide exposure imposing a selection pressure as a consequence of CLS management, recovered pathogen populations were already predominated by resistant

Table 3. Comparison of resistance factors (RFs) in *Alternaria* spp. populations recovered in 2016 and 2017 from Upper Great Lakes sugarbeet production^a

Year	Pyraclostrobin				Triphenyl tin OH			
	RS ^b	MI	I	R	RS	MI	I	R
2016	8.8	46.2	108.4	202.9	12.4	28.4	86.7	–
2017	6.9	37.9	115.7	201.4	10.1	46.2	98.6	185.7

^a Values are presented as RFs, which were calculated as the ratio of the mean EC_{50} value of *Alternaria* spp. populations displaying decreased sensitivity compared with the mean EC_{50} for reference sensitive populations.

^b Sensitivity of isolates to pyraclostrobin and triphenyl tin OH was categorized as follows: RS = reduced sensitivity ($EC_{50} = 1$ to 10 mg/liter), MI = moderately insensitive ($EC_{50} = 10$ to 50 mg/liter), I = insensitive ($EC_{50} = 50$ to 100 mg/liter), and R = resistant ($EC_{50} > 100$ mg/liter).

Table 2. Comparison of resistance factors (RFs) in *Alternaria* spp. populations recovered in 2016 and 2017 from Upper Great Lakes sugarbeet production^a

Year	Difenoconazole				Fenbuconazole				Flutriafol				Prothioconazole				Tetraconazole			
	RS ^b	MI	I	R	RS	MI	I	R	RS	MI	I	R	RS	MI	I	R	RS	MI	I	R
2016	6.3	35.3	–	–	7.0	48.5	101.7	186.9	6.8	40.6	83.2	163.8	7.4	39.6	100.3	188.1	6.9	48.3	103.9	172.7
2017	4.8	32.6	105.4	143.4	9.0	44.5	111.7	187.2	6.1	38.2	88.8	181.6	5.8	40.5	103.4	187.0	8.0	36.0	83.1	177.3

^a Values are presented as RFs, which were calculated as the ratio of the mean EC_{50} value of *Alternaria* spp. populations displaying decreased sensitivity compared with the mean EC_{50} for reference sensitive populations.

^b Sensitivity of isolates to five demethylase inhibitor fungicides (difenoconazole, fenbuconazole, flutriafol, prothioconazole, and tetraconazole) was categorized as follows: RS = reduced sensitivity ($EC_{50} = 1$ to 10 mg/liter), MI = moderately insensitive ($EC_{50} = 10$ to 50 mg/liter), I = insensitive ($EC_{50} = 50$ to 100 mg/liter), and R = resistant ($EC_{50} > 100$ mg/liter).

individuals. In previous studies on *Alternaria* late blight (ALB) of pistachio in California, caused by *Alternaria* spp. in the *A. alternata*, *A. tenuissima*, and *A. arborescens* species groups, populations of *A. alternata* contained a mixture of isolates varying in DMI sensitivity (Avenot et al. 2016) and variations in EC₅₀ distributions for the three DMIs tested in the current study were consistent with other patterns of DMI sensitivity in various fungi (Brent and Hollomon 1995). The ranges of sensitivities to tebuconazole and propiconazole were relatively wider within the *A. alternata* populations compared with that observed for difenoconazole (Avenot et al. 2016). Similarly, the recorded variations in *A. alternata* sensitivity to tebuconazole and the two other DMIs (propiconazole and difenoconazole) were within the range of the natural sensitivities of this pathogen to these fungicides (Avenot et al. 2016). The ranges of EC₅₀ values were broad and very similar for both tebuconazole and propiconazole, and there were differences in intrinsic activity of the three DMIs tested; difenoconazole appeared more active than the two other fungicides in inhibiting *A. alternata* mycelial growth with a narrower distribution of EC₅₀ values, similar to Avenot et al. (2016). In studies on *A. alternata* causing moldy core in apple, among the tested chemicals, polyoxin B and trifloxystrobin (QoI) had the strongest effect on germination of conidia; a much higher concentration was needed to achieve the same degree of germination inhibition by azoxystrobin (QoI) or difenoconazole (DMI) (Reuveni and Sheglov 2002). Among tested chemicals, mycelial growth of the fungus was most sensitive to difenoconazole, which was effective at 0.8–12 mg/liter; at higher concentrations, polyoxin B, was effective at suppressing mycelial growth, whereas the strobilurins (trifloxystrobin and azoxystrobin) were less effective, with EC₉₅ values of >1,000 mg/liter (Reuveni and Sheglov 2002). Similar studies on blackspot of crucifers, caused by *Alternaria* spp., found three sterol biosynthesis-inhibiting fungicides; two of the triazole group (flutriafol and difenoconazole) and one of the imidazole group (prochloraz), performed equally well in reducing the mycelial growth of *A. brassicae*, *A. brassicicola*, and *A. japonica* (Iacomi-Vasilescu et al. 2004). In blackspot of crucifers, isolates of *Alternaria* spp. were separated into four classes depending on their sensitivity to tested fungicides (iprodione, procymidone, difenoconazole, flutriafol, prochloraz, and fludioxonil): isolates were classified as sensitive, with

EC₅₀ values ranging between 0.1 and 1 mg/liter; weakly resistant, with EC₅₀ values between 1 and 10 mg/liter; intermediate resistant, with EC₅₀ values between 10 and 100 mg/liter; and highly resistant, with EC₅₀ values above 100 mg/liter (Iacomi-Vasilescu et al. 2004). The dicarboximide iprodione proved to be more effective than procymidone with most isolates sensitive to the phenylpyrroles fludioxonil with some *A. brassicicola* isolates highly resistant (EC₅₀ >100 mg/liter) to both the dicarboximides iprodione and procymidone and phenylpyrroles (e.g., fludioxonil) (Iacomi-Vasilescu et al. 2004).

In the current study, the response to tetraconazole in *Alternaria* spp. related to a decrease in mean EC₅₀ also was reflected in a decrease in the RF for both MI and I sensitivity phenotypes. Similarly, mean EC₅₀ values among *Alternaria* spp. from sampled counties and provinces increased from 2016 to 2017. Overall, RFs for the I and R phenotypes increased for four of the fungicides (difenoconazole, fenbuconazole, flutriafol, and triphenyl tin OH), although the RFs for the R class decreased for the two fungicides (prothioconazole and pyraclostrobin). The population response to fungicides based on RFs taken together with mean sensitivities suggests a shift in the response of populations of *Alternaria* spp. causing ALS in the Upper

Table 6. Maximum-likelihood analysis of variance from log-linear analysis for the main effects of year on the frequency of sensitivity categories of *Alternaria* spp. recovered from Michigan and Ontario sugarbeet production

Sensitivity ^a	df	Pyraclostrobin			df	Triphenyl tin OH		
		χ^2	$P > \chi^2$ ^b	Estimate		χ^2	$P > \chi^2$	Estimate
S	1	0.07	0.7964	0.0668	—	—	—	—
RS	—	—	—	—	1	1.82	0.1772	-0.4055
MI	1	0.24	0.6227	-0.0607	1	1.11	0.2918	0.0697
I	—	—	—	—	—	—	—	—
R	1	23.98	<0.0001	0.4686	—	—	—	—

^a Sensitivity of isolates to pyraclostrobin and triphenyl tin OH was categorized as follows: S = sensitive (EC₅₀ < 1 mg/liter), RS = reduced sensitivity (EC₅₀ = 1 to 10 mg/liter), MI = moderately insensitive (EC₅₀ = 10 to 50 mg/liter), I = insensitive (EC₅₀ = 50 to 100 mg/liter), and R = resistant (EC₅₀ > 100 mg/liter).

^b Significant if $P < 0.05$.

Table 4. Maximum-likelihood analysis of variance from log-linear analysis for the effects of year and county on the frequency of sensitivity categories of *Alternaria* spp. recovered from Michigan and Ontario sugarbeet production

Sensitivity ^a	Difenoconazole			Fenbuconazole			Flutriafol			Prothioconazole			Tetraconazole		
	df	χ^2	$P > \chi^2$ ^b	df	χ^2	$P > \chi^2$	df	χ^2	$P > \chi^2$	df	χ^2	$P > \chi^2$	df	χ^2	$P > \chi^2$
S	1	37.00	<0.0001	1	0.14	0.7064	1	0.32	0.5714	1	8.86	0.0029	—	—	—
RS	1	15.92	<0.0001	1	45.86	<0.0001	1	1.28	0.2585	1	7.20	0.0073	1	0.60	0.4369
MI	1	9.76	0.0018	1	0.01	0.9042	1	3.4	0.0650	1	0.37	0.5417	1	3.12	0.0773
I	1	0.08	0.7782	1	1.28	0.2571	1	15.11	0.0001	1	1.43	0.2324	1	2.70	0.1001
R	1	15.37	<0.0001	—	—	—	1	5.63	0.0177	1	2.31	0.1285	1	4.35	0.0371

^a Sensitivity of isolates to difenoconazole, fenbuconazole, flutriafol, prothioconazole, and tetraconazole was categorized as follows: S = sensitive (EC₅₀ < 1 mg/liter), RS = reduced sensitivity (EC₅₀ = 1 to 10 mg/liter), MI = moderately insensitive (EC₅₀ = 10 to 50 mg/liter), I = insensitive (EC₅₀ = 50 to 100 mg/liter), and R = resistant (EC₅₀ > 100 mg/liter).

^b Significant if $P < 0.05$.

Table 5. Analysis of maximum-likelihood estimates from log-linear analysis for the main effects of year on the frequency of sensitivity categories of *Alternaria* spp. recovered from Michigan and Ontario sugarbeet production

Sensitivity ^a	Difenoconazole		Fenbuconazole		Flutriafol		Prothioconazole		Tetraconazole	
	Estimate	$P > \chi^2$ ^b	Estimate	$P > \chi^2$	Estimate	$P > \chi^2$	Estimate	$P > \chi^2$	Estimate	$P > \chi^2$
S	0.9423	<0.0001	0.1438	0.7064	-0.3466	0.5714	0.6365	0.0029	—	—
RS	0.6496	<0.0001	1.1256	<0.0001	0.1438	0.2585	0.4581	0.0073	0.6798	0.4369
MI	-0.6264	0.0018	-0.0145	0.9042	-0.2197	0.0650	0.0748	0.5417	0.2145	0.0773
I	-0.1733	0.7782	0.2695	0.2571	0.8047	0.0001	0.3031	0.2324	0.2853	0.1001
R	-1.1836	<0.0001	—	—	0.2682	0.0177	-0.1465	0.1285	-0.1705	0.0371

^a Sensitivity of isolates to difenoconazole, fenbuconazole, flutriafol, prothioconazole, and tetraconazole was categorized as follows: S = sensitive (EC₅₀ < 1 mg/liter), RS = reduced sensitivity (EC₅₀ = 1 to 10 mg/liter), MI = moderately insensitive (EC₅₀ = 10 to 50 mg/liter), I = insensitive (EC₅₀ = 50 to 100 mg/liter), and R = resistant (EC₅₀ > 100 mg/liter).

^b Significant if $P < 0.05$.

Great Lakes. Resistance to strobilurins has been demonstrated extensively in previous studies for several *Alternaria* spp., including *Alternaria* from sugarbeet (Avenot et al. 2008; Ma and Michailides 2004b; Malandrakis et al. 2015; Pasche et al. 2005; Rosenzweig et al. 2008a, b, 2017). Previous DNA-based and sequencing analysis revealed that all intermediate and resistant isolates contained the G143A mutation, which confers QoI resistance in *Alternaria* spp. (Rosenzweig et al. 2017). In the case of ALB on tomato, *A. alternata* isolates with dual resistance to QoIs (pyraclostrobin and azoxystrobin) were identified (Avenot et al. 2008).

For the triphenyltin class of fungicides, *C. beticola* isolates resistant to triphenyltin hydroxide have been reported in Europe and the United States (Bugbee 1995; Giannopolitis 1978; Giannopolitis and Chrysai-Tokousbalides 1980; Karaoglanidis and Iannidis 2010; Marić et al. 1984; Secor et al. 2010a, b). In fungicide sensitivity monitoring of sugarbeet production in Greece, an increase in the frequency of tin resistance in *C. beticola* was regionally specific. Additionally, in Greece and the United States, the frequency of tin resistance in *C. beticola* was significantly reduced after a reduction or elimination of tin fungicide applications or fungicide use history (Giannopolitis 1978; Ioannidis 1994; Secor et al. 2010b). Restricted tin fungicide use allowed the reduction, later in the season, of the resistant strains selected for by multiple tin applications, since in the absence of tin fungicides the resistant strains do not compete well with the sensitive strains (Giannopolitis and Chrysai-Tokousbalides 1980). To our knowledge, the current study is the first report of tin tolerance in a fungus other than *C. beticola*, with isolates collected in 2017 that had EC₅₀ values >100 mg/liter (classified as resistant).

Sugarbeet leaf spot disease management issues related to persistent applications of fungicides for CLS likely has led to the development of insensitivity in *Alternaria* spp. to the QoI, DMI, and triphenyltin hydroxide fungicides. This fungicide insensitivity coincided with an increase incidence and severity of ALS observed at levels that cause yield loss as a result of defoliation and reduction in photosynthetic capacity. These results indicate that few if any of these currently used fungicides for leaf spot disease management in sugarbeet may be effective at controlling many *Alternaria* isolates found in the region. Moreover, individual isolates were recovered with multiple resistance to two or even all three of the fungicide classes. Determining the efficacy of currently labeled fungicides for CLS management in sugarbeet for use in ALS disease management, coupled with fungicide resistance monitoring, is essential for developing use recommendations for the region. This current study indicates the potential for *Alternaria* spp. populations that have multiple resistance and predisposition to reduced sensitivity to three of the major fungicides labeled for management of leaf spot on sugarbeet. Results are similar to those for other crops. For example, the distribution of sensitivities to DMI fungicides among isolates in *A. alternata* populations causing ALB in California was continuous, ranging from sensitive phenotypes to phenotypes with reduced sensitivity to tebuconazole, propiconazole, and, to a lesser extent, difenoconazole (Avenot et al. 2016). Cross-resistance among these three DMIs also was found but varied owing to differences in sensitivity patterns among fungal isolates and the relative intrinsic activity of DMIs (Gubler et al. 1996; Leroux and Walker 2011). Cross-resistance among fungicides within the DMI class has been reported in other fungi as well (Karaoglanidis and Thanassouloupoulos 2003; Köller et al. 1997; Thomas et al. 2012). Cross-resistance was observed among the DMI fungicides tebuconazole, difenoconazole, and propiconazole in *A. alternata* from pistachio. This is common among fungicides belonging to the same chemical class because they share a similar mode of action, and this has implications for disease management using DMIs (Avenot et al. 2016). The higher intrinsic activity shown by difenoconazole suggests that in the event of poor control with one DMI (e.g., tebuconazole), the use of this fungicide in the field could provide adequate control of ALB (Avenot et al. 2016), and the current study indicates that a similar situation may be true for ALS on sugarbeet.

Continued monitoring of populations of *Alternaria* spp. causing ALS in the Upper Great Lakes is required to determine the sensitivity to available fungicides and determine baseline sensitivity to new

chemistries. Toward determining baseline sensitivity to existing fungicides, the need to explore sources of nonfungicide exposed isolates to have a better estimate of the shifts in sensitivity is essential. Potentially a source of baseline isolates could be from organic table beet production fields. The development of DNA-based approaches targeting mutations responsible for fungicide resistance will aid in the early detection of resistant individuals. Additionally, studies on the competitive fitness of individuals resistant to tin or resistant to multiple fungicide modes of action and their impact on disease management are needed. An integrated approach, combining sensitivity monitoring with fungicide efficacy related to pathogen biology, is essential to developing fungicide resistant management recommendations. For example, because these results indicate a significant potential for cross-resistance between related DMI fungicides, it is recommended to not use DMIs successively, limit the total number of applications of DMIs in a season, and alternate or combine fungicide chemistries with different modes of action. In addition, the development of fungicide resistance management recommendations for *Alternaria* spp. should incorporate epidemiological models that focus on application tactics, fungicide dynamics, effects of fungicide, and dose response. Epidemiological models of application tactics should compare the efficacy of mixtures to alternations (e.g., high-low versus low-high). Moreover, the resistant management models should integrate previously validated leaf spot disease prediction models (Bolton et al. 2012; Kaiser et al. 2010; Khan et al. 2007; Windels et al. 1998) adapted over successive growing seasons. Such models would need to be adapted for ALS, since they were developed for leaf spot from *C. beticola*, which has higher optimum temperatures for disease development, between 25 and 35°C (Franc 2010) and disease development occurring up to 40°C (Jacobsen and Franc 2009), whereas ALS has lower optimum temperatures. The effects of fungicide sequence and timing should be evaluated for fungicides considered high risk (e.g., pyraclostrobin), moderate risk (e.g., difenoconazole), and low risk (e.g., mancozeb) for resistance development. In addition to development of resistance management recommendations for use of currently available fungicides labeled for leaf spot of sugarbeet, the use of ALS resistant cultivars can potentially enhance and prolong fungicide efficacy. However, the availability of resistant cultivars is not known, as there has been little testing for response to this disease in beet. The development of new chemistries and evaluating efficacy and sensitivity of *Alternaria* spp. populations to available chemistries not currently labeled for leaf spot of sugarbeet will be essential for potential fungicide programs going forward.

Acknowledgments

The authors thank all of the agriculturists from Michigan Sugar Company who assisted in collection of samples.

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