Population Diversity and Sensitivity to Azoxystrobin of *Alternaria brassicicola* in New York State

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Abstract

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Alternaria brassicicola is the causal agent of Alternaria leaf spot, a common disease of brassica crops in New York State. New York isolates of *A. brassicicola* were collected from a variety of brassica crops and locations to evaluate the population diversity and screen for fungicide sensitivity. Isolates were genotyped for 10 microsatellite loci and assayed for sensitivity to azoxystrobin, a quinone outside inhibitor fungicide. The New York State population of *A. brassicicola* was found to have high levels of genotypic diversity

and the population was found to be in linkage disequilibrium. Based on in vitro assays, the effective concentrations of azoxystrobin reducing spore germination by 50% ranged from 0.22 to 14.12 $\mu\text{g/ml}$. In order to confirm the sensitivity of 47 isolates to azoxystrobin, the *cytb* gene was characterized and sequenced to determine whether any of the mutations known to confer resistance to azoxystrobin were present. The mutations F129L, G137R, and G143A were not detected in the isolates studied.

New York State is the second largest producer of fresh-market cabbage (*Brassica oleracea* var. *capitata*) in the United States, with 8,300 acres harvested in 2014 for a value of \$72 million (USDA 2015). New York producers also grow fresh-market cauliflower (*B. oleracea* var. *botrytis*) and processing cabbage along with many other minor brassica crops intended for the fresh market. An economically important pathogen for cultivated brassica crops is *Alternaria brassicicola* (Schwein.) Wiltshire, causal agent of Alternaria leaf spot. The disease begins with small black lesions (1 mm) on the leaves that enlarge over time to form concentric bull's eye lesions. The disease can appear on the harvested portion of the crop, resulting in a damaged and unmarketable crop, and is especially noticeable in cauliflower curds, where black spots appear on the white florets.

The climate of New York State is an ideal environment for *A. brassicicola* which has optimal sporulation at temperatures between 20 to 30°C and a relative humidity of at least 87% (Humpherson-Jones and Phelps 1989). *A. brassicicola* epidemics can also start the following growing season due to seed transmission and survival on crop debris (Humpherson-Jones 1989; Köhl et al. 2011, 2010). *A. brassicicola* produces conidia that are dispersed by wind and rain splash (Chen et al. 2003). Infection can also spread during the growing season through insects and weed species (Cobb and Dillard 1998; Dillard et al. 1998).

A. brassicicola is thought to reproduce primarily through asexual reproduction. Although no sexual stage has been found, high levels of diversity have been observed in *A. brassicicola* populations in Australia (Bock et al. 2005; Linde et al. 2010). The authors suggested that recombination was occurring occasionally for *A. brassicicola*. Bock et al. (2005) found high levels of genetic diversity for five populations of *A. brassicicola* in Australia infecting *Cakile maritima* using amplified fragment length polymorphisms indicative of a randomly mating population; however, the populations studied were in linkage disequilibrium, suggesting that some clonality exists among

the populations. Linkage disequilibrium is a measurement of the non-random association of alleles at different loci; however, high levels of genotypic diversity along with gene diversity can be an indication of recombination (Milgroom 1996).

Another study used microsatellites to look at *A. brassicicola* populations from *C. maritima* in Australia and also found high levels of genetic diversity and gene diversity (Linde et al. 2010). Similarly, other studies on *Alternaria* spp. have also found high levels of diversity, including *A. alternata* on pear, pistachio, potato, tomato, and citrus along with indoor allergen samples (Adachi et al. 1993; Aradhya et al. 2001; Meng et al. 2015a; Morris et al. 2000; Stewart et al. 2013; Woudenberg et al. 2015). High levels of diversity have also been found in *A. solani* on tomato and potato (Meng et al. 2015b; van der Waals et al. 2004). However, little was known about the diversity of the upstate New York population of *A. brassicicola*.

Growers in New York State commonly use strobilurin fungicides such as azoxystrobin to control for Alternaria leaf spot, making one or two sprays per season during peak disease in the fall. There have been no obvious control failures; however, there is always concern about the potential for resistant isolates to be present in the pathogen population. Azoxystrobin, a quinone outside inhibitor (QoI) fungicide, has a single-site mode of action. Azoxystrobin inhibits mitochondrial respiration by binding to the Qo site of cytochrome *b* (*cytb*), blocking electron transfer between *cytb* and cytochrome *c* and, thus, disrupting the energy cycle by prohibiting ATP production (Bartlett et al. 2002). Resistance to azoxystrobin has been found in *A. alternata*, *A. tenuissima*, *A. arborescens*, and *A. solani* (Ma and Michailides 2004; Ma et al. 2003; Pasche et al. 2004). Three single-site mutations in gene *cytb* (F129L, G137R, and G143A) have been found in agricultural systems that confer resistance to azoxystrobin (Fisher and Meunier 2008). The F129L mutation confers partial resistance and replaces a phenylalanine with a leucine at codon 129 in the *cytb* gene and has been found in *A. solani* (Pasche et al. 2005). The G143A mutation confers complete resistance and replaces a guanine with an alanine at *cytb* codon 143 and has been found in various *Alternaria* spp. (Ma et al. 2003; Vega et al. 2012). The G143 mutation is much more prevalent and the cause of the QoI resistance in many plant pathogens (Gisi et al. 2002). The G137R mutation is less common but has been found to confer partial resistance by exchanging a glycine with an arginine at *cytb* codon 137 (Sierotzki et al. 2007). The goal of this study is to determine how diverse the population of *A.*

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brassicicola is for New York State and to determine the level of sensitivity of New York isolates of *A. brassicicola* to azoxystrobin.

Materials and Methods

Isolates used in this study. In total, 49 *A. brassicicola* isolates were collected over a period of years and locations across New York State (Table 1). Small pieces of plant tissue from the edge of a leaf lesion were disinfested and rinsed in sterile distilled water before plating on potato dextrose agar (Difco PDA; Becton, Dickinson, and Company, Franklin Lakes, NJ). Single conidial isolates were obtained from each culture with standard dilution procedures.

DNA extraction and confirmation of *A. brassicicola* isolates. Small amounts of mycelia from the single conidial isolates were scraped off the agar in each plate using a sterile pipette tip and were placed in 50 ml of potato dextrose broth in 200-ml Erlenmeyer flasks and grown for 5 to 10 days at room temperature (22°C) on a shaker at 100 rpm. The mycelium was then collected by vacuum filtration. DNA extractions were performed using the Qiagen DNeasy Plant Mini Kit according to the manufacturer's instructions, with slight modifications (Qiagen, Valencia, CA). In order to disrupt the tissue, 35 to 100 mg of wet mycelia was placed in a 2-ml safe-lock, round-bottom centrifuge tube with one 5-mm sterile stainless steel grinding bead (Qiagen) and shaken in a Qiagen Tissue-Lyser at 30 cycles per second for 30 s. After the final wash step with buffer AW2, a 1-min centrifuge cycle was added to help dry the filter. The incubation with AE elution buffer was extended to 15 min for the final step of the kit protocol (Dunn et al. 2010). Quantity and quality of the extracted DNA was determined using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE).

Each single conidial isolate was confirmed to be *A. brassicicola* with polymerase chain reaction (PCR) using previously designed species-specific primers ABRA1 and ABRA2 (Iacomi-Vasilescu et al. 2002). Each 25- μ l reaction contained 1 μ l of DNA (10 to 50 ng), 0.2 μ M each primer, 200 μ M each dNTP, 1 $\times$ Green GoTaq reaction buffer containing 1.5 mM MgCl₂, and 1.0 U of GoTaq DNA polymerase (Promega Corp., Madison, WI). PCR assays were conducted in a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA) with an initial denaturation of 3 min at 94°C; followed by 35 cycles of 30 s at 94°C, 50 s at 55°C, and 1 min at 72°C; and a final elongation at 72°C for 10 min. PCR assays were verified by running on a 1.5% agarose gel with 1% ethidium bromide. Each isolate was also tested with primers ABRE1 and ABRE3 previously developed for *A. brassicae*, which has a similar appearance to *A. brassicicola* in culture (Iacomi-Vasilescu et al. 2002). The same PCR conditions that were used for the ABRA1/ABRA2 primer pair were also used for the ABRE1/ABRE3 primer pair.

Simple-sequence repeat analyses. Ten microsatellite loci were used to determine genotypes for 49 *A. brassicicola* isolates using primer pairs previously designed for *A. brassicicola* (Avenot et al. 2005). Forward primers for each pair were fluorescently labeled with one of the following dyes at the 5' end: 6-FAM, VIC, NED, or PET (Applied Biosystems, Grand Island, NY). Primer pairs were used in separate 25- μ l PCR assays containing 10 ng of DNA, 0.4 μ M each primer, 200 μ M each dNTP, 1 $\times$ Green GoTaq reaction buffer containing 1.5 mM MgCl₂, and 1.0 U of GoTaq DNA polymerase (Promega Corp.). PCR assays were conducted in a Bio-Rad C1000 Touch Thermal Cycler with an initial denaturation of 3 min at 94°C; followed by 30 cycles of 30 s at 94°C, 30 s at the appropriate annealing temperature (see below), and 30 s at 72°C; with a final elongation at 72°C for 10 min. Primers for loci Abmic1, Abmic2, Abmic3, Abmic4, Abmic5, and Abmic7 were amplified with an annealing temperature of 55°C. Loci Abmic9 and Abmic11 were amplified with an annealing temperature of 60°C, Abmic8 at 58°C, and Abmic10 at 56°C (Avenot et al. 2005). For analysis, 1 μ l of PCR product was added to 0.04 μ l of GeneScan-500 LIZ size standard (Applied Biosystems) and 15 μ l of formamide (Sigma-Aldrich, St. Louis). Fragments were sized using an ABI 3730xl DNA Analyzer at the Cornell University Institute of Biotechnology Genomic Facility. Electropherograms were viewed in PeakScanner (Applied Biosystems) and alleles were scored manually.

Statistical analysis. The RStudio statistical software, version 0.99.878 (RStudio Team 2015), was used along with the *poppr*

package, version 2.1.0 (Kamvar et al. 2014), to determine the number of multilocus genotypes (MLG) in the 49 isolates using allele sizes from the 10 loci assayed. An analysis of molecular variance (AMOVA) was conducted using *poppr* to estimate the proportion of variation observed from differences within and among counties, years, and isolates from different host plants (Excoffier et al. 1992). Significance was determined using 999 permutations. The *poppr* package was used to create a dendrogram of all isolates using the unweighted pair-group method with arithmetic mean analysis and Bruvo's distance with 999 bootstraps and cutoff value of 50%. Minimum spanning networks were also created for host, county, and year collected using Bruvo's distance. The *poppr* package was also used to calculate

Table 1. Sensitivity of New York State isolates of *Alternaria brassicicola* with the year they were collected, the county where they were collected, the host plant from which they were isolated, and the concentration of azoxystrobin that effectively reduced spore germination by 50% (EC₅₀)

Isolate	Year collected	County	Host plant	EC ₅₀ (μ g/ml) ^a
759B	1994	Unknown	Cabbage	0.75
914A	1997	Ontario	Pennycress	2.11
955A	2000	Unknown	Cabbage	2.25
990A	2001	Yates	Cabbage	2.23
997A	2002	Dutchess	Cabbage	7.78
1002A	2002	Cortland	Cabbage	14.12
1121A	2010	Ontario	Cabbage	1.72
1122A	2010	Orleans	Cabbage	2.29
1123A	2010	Wayne	Cabbage	4.61
1126A	2010	Ontario	Cabbage	6.71
1128A	2010	Ontario	Cauliflower	–
1130A	2010	Ontario	Cauliflower	0.74
1133A	2010	Orleans	Cabbage	1.02
1135A	2010	Monroe	Cauliflower	7.76
1136A	2010	Monroe	Cabbage	0.61
1138A	2010	Ontario	Cabbage	5.59
1139A	2010	Ontario	Cabbage	0.60
1140A	2010	Ontario	Cabbage	0.65
1141A	2010	Ontario	Cabbage	0.86
1144A	2011	Ulster	Kale	0.86
1146A	2011	Suffolk	Brussels sprouts	0.43
1147A	2011	Ontario	Cauliflower	2.33
1149A	2011	Ontario	Broccoli	0.97
1153A	2011	Rensselaer	Cabbage	1.12
1155A	2011	Ontario	Cabbage	1.04
1156A	2011	Monroe	Cabbage	1.43
1157A	2011	Ontario	Cabbage	0.33
1158A	2011	Ontario	Cabbage	7.12
1160A	2011	Ontario	Kale	1.56
1161A	2011	Washington	Cabbage	2.79
1162A	2011	Monroe	Brussels sprouts	2.70
1163A	2011	Orleans	Cabbage	0.22
1164A	2011	Orleans	Cabbage	1.45
1165A	2011	Genessee	Broccoli	0.49
1166A	2011	Genessee	Cauliflower	0.66
1167A	2011	Genessee	Cabbage	0.64
1168A	2011	Tompkins	Brussels sprouts	0.99
1169A	2011	Tompkins	Brussels sprouts	11.03
1170A	2011	Tompkins	Cabbage	1.89
1171A	2011	Tompkins	Kale	0.57
1172A	2011	Tompkins	Kale	0.92
1173A	2011	Tompkins	Cabbage	1.11
1193A	2013	Ontario	Cabbage	0.28
1195A	2013	Genessee	Brussels sprouts	2.68
1196A	2013	Genessee	Cauliflower	1.56
1198A	2013	Genessee	Broccoli	2.16
1199A	2013	Erie	Broccoli	5.13
1200A	2013	Ulster	Broccoli	–
14001A	2014	St Lawrence	Cabbage	0.29

^a EC₅₀ was calculated using a probit analysis; – indicates isolates that were included in simple-sequence repeat analysis; however, they were lost prior to the azoxystrobin sensitivity screening.

the following for the entire New York population: Shannon-Wiener's index of genetic diversity (H') (Shannon 2001), Stoddart and Taylor's index of MLG diversity (G) (Stoddart and Taylor 1988), Simpson's index of diversity (λ) (Simpson 1949), evenness (E_5) (Grünwald et al. 2003), and the index of association (I_A) (Brown et al. 1980). Nei's index of gene diversity (H_{exp}) (Nei 1978) was also calculated after removing any nonpolymorphic loci.

Azoxystrobin in vitro sensitivity assay. Isolates were exposed to azoxystrobin (Sigma-Aldrich) by adding the chemical (dissolved in acetone) along with salicylic hydroxamic acid (SHAM; Sigma-Aldrich) dissolved in methanol to sterile water agar (1.5%) that had been cooled to 55°C (Ma et al. 2003; Pasche et al. 2004). The final concentrations of azoxystrobin active ingredient (a.i.) in the water agar media were 25, 10, 1.0, 0.1, 0.01, and 0.001 μg a.i./ml, while all media contained SHAM at 60 μg /ml (Vaghefi et al. 2016). Final concentrations of the solvents in the fungicide amended water agar did not exceed 0.25% (vol/vol). Control plates consisted of water agar and water agar amended with SHAM at 60 μg /ml.

A. brassicicola isolates were tested for fungicide sensitivity to azoxystrobin by determining the effective concentration at which 50% spore germination was inhibited (EC_{50}). A spore suspension was made from a 7-day-old culture grown on PDA at room temperature with 15 h of daylight by adding 5 ml of sterile distilled water to the culture and dislodging spores with a sterile, L-shaped spreader. The suspension was then passed through a double layer of sterile cheesecloth. Concentration of spore suspension was determined with a hemocytometer and adjusted to 10^4 conidia/ml. The suspension (50 μl) was spread onto water agar amended with fungicide and SHAM. Plates were stored in darkness for 24 h at room temperature. After 24 h, 100 conidia were counted per plate by placing agar squares on a slide, and examined microscopically. A conidium was considered germinated if the germ tube was longer than or equal in length to the conidium (Rogers and Stevenson 2010). The entire experiment was repeated, for a total of two plates (100 conidia/plate) that were counted for each isolate at each fungicide concentration along with the water agar and water agar plus SHAM controls.

The Statistical Analysis System (SAS, version 9.4; Cary, NC) was used to perform a t test across all isolates comparing the water agar control and the water agar plus SHAM control to determine whether the SHAM resulted in any germination inhibition. A probit analysis was performed using SAS to calculate the EC_{50} based on the water agar control.

***cytb* Sequencing.** Primers were developed for the *A. brassicicola* *cytb* gene targeting the hotspots for mutations known to confer azoxystrobin resistance, F129L and G143A. Two sets of primers were designed due to a large intron (approximate 1,315 bp) located after codon 131. Primers CytB129.2F (5'-AAC CCA TAG ATA GTG CGT CTA GGA-3') and CytB129.2R (5'-ATC TAC GCG GAG GTG ATA CAG-3') were used to amplify a fragment of the *cytb* gene

containing codon 129 and primers AbCytB101F (5'-TTG GCG ATG TTA GTG AAT ACG A-3') and AbCytB92R (5'-ATA CCC ATC TCC ATC AAT TAG TCC-3') were used to amplify a fragment of the *cytb* gene containing codons 137 and 143. For these PCR amplifications, each 25- μl reaction contained 30 ng of genomic DNA, 0.2 μM each primer, and 1 $\times$ EmeraldAmp GT PCR Master Mix (Takara Clontech Laboratories, Mountain View, CA). PCR assays were conducted in a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad) with an initial denaturation of 3 min at 94°C; followed by 35 cycles of 30 s at 94°C, 30 s at 59°C, and 30 s at 72°C; and a final elongation at 72°C for 10 min. PCR assays were verified by running on a 1.5% agarose gel with 1% GelRed Nucleic Acid Stain (Biotium, Hayward, CA). PCR products were then purified using Zymo DNA Clean and Concentrator kit (Zymo Research Corp., Irvine, CA), and sequenced at the Cornell University Institute of Biotechnology Genomic Facility. Sequences were analyzed using Molecular Evolutionary Genetics Analysis (Tamura et al. 2013).

Results

Simple-sequence repeats genotyping. In total, 49 isolates collected in New York State were used in the diversity study (Table 1). In all, 8 of the 10 microsatellite loci examined were found to be polymorphic, with a total of 44 MLG observed. The number of alleles found for each of the loci ranged from 1 to 10 alleles (Table 2). In all cases, one allele was amplified per isolate per locus. Five of the MLG were identified twice. In four of the five shared MLG, there was a common host, county, or year of collection (Table 3). Results from the AMOVA revealed that there was no significant genetic differentiation between or within counties and years of collection. The AMOVA did reveal significant genetic differentiation ($P < 0.05$) among isolates from different host plants and accounted for 4.36% of the variation in the New York population. Most of the variation observed occurred within samples.

The dendrogram and minimum spanning networks revealed little structure because no clustering was observed by host, county, or year of collection. There were only eight bootstrap values greater than 50% identified (data not shown). Measurements for genotypic diversity were high as determined by H' , G , and λ (Table 4). H_{exp} was high for the entire population (Table 4). The genotypes occur at approximately the same frequency in the population given the high E_5 (Table 4). The I_A was significant ($P > 0.05$), indicating that the population observed is in linkage disequilibrium.

Sensitivity to azoxystrobin and *cytb* sequencing. No significant differences were observed between the water agar control and the water agar plus SHAM control. For the 47 isolates tested, the EC_{50} values for azoxystrobin ranged from 0.22 to 14.12 a.i. μg /ml, with a mean EC_{50} value of 2.49 μg /ml. Of the 47 isolates, 19 were highly sensitive (EC_{50} value < 1.0 μg /ml) and 20 of the 47 isolates were moderately sensitive (< 5.0 μg /ml). The remaining eight isolates were less sensitive, with EC_{50} values of 5.13 to 14.12 μg /ml. Due to the range of EC_{50} values, partial sequences for the *cytb* gene were obtained for all isolates tested with azoxystrobin. Mutations known to confer resistance to azoxystrobin were not found in any of the 47 isolates studied. All of the 47 isolates were found to have a large intron (approximately 1,315 bp). No heteroplasmy was observed in

Table 2. Number of alleles detected for *Alternaria brassicicola* isolates for each microsatellite locus

Microsatellite marker ^a	N ^b	H _{exp} ^c	Size of alleles (bp)
Abmic1	2	0.12	115, 117
Abmic2	2	0.33	124, 126
Abmic3	3	0.53	105, 107, 109
Abmic4	6	0.62	76, 92, 94, 96, 115, 135
Abmic5	1	-	97
Abmic7	1	-	107
Abmic8	5	0.66	146, 148, 150, 152, 156
Abmic9	9	0.80	76, 90, 92, 94, 96, 98, 100, 102, 104
Abmic10	2	0.38	151, 154
Abmic11	10	0.85	180, 183, 185, 188, 191, 197, 200, 206, 209, 212

^a Microsatellite markers developed by Avenot et al. (2005).

^b Number of alleles for individual microsatellite loci.

^c Nei's index of gene diversity (H_{exp}) = $1 - \sum p_i^2$, where p_i is the frequency of the i th allele at the given locus (Nei 1978).

Table 3. Collection details for the *Alternaria brassicicola* isolates that had the same multilocus genotypes (MLG) as determined by 10 microsatellite loci

MLG	Isolate	Year	County	Host
MLG 5	1136A	2010	Monroe	Cabbage
	1141A	2010	Ontario	Cabbage
MLG 6	1167A	2011	Genessee	Cabbage
	1198A	2013	Genessee	Broccoli
MLG 14	1169A	2011	Tompkins	Brussels Sprouts
	1193A	2013	Ontario	Cabbage
MLG 27	990A	2001	Yates	Cabbage
	1138A	2010	Ontario	Cabbage
MLG 37	1135A	2010	Monroe	Cauliflower
	1196A	2013	Genessee	Cauliflower

the electropherograms for any of the isolates at the locations for the three known mutations.

Discussion

This study revealed that the New York State population of *A. brassicicola* has high genotypic diversity as well as high gene diversity. Of 49 isolates, 44 unique MLG were found and confirmed by indices of genotypic diversity. The mean gene diversity, which is calculated using the number and frequencies of alleles at each locus, was also high, with an estimate of 0.53 for the population. High levels of genotypic diversity is one of the major outcomes of having a recombinant population, along with random association between alleles at different loci (Milgroom 1996). The New York State population had high levels of diversity; however, the I_A calculated for the New York population was significantly different from zero, indicating linkage disequilibrium and, thus, a nonrandom association of loci. The I_A indicates that asexual reproduction is important for the population. A population can be in linkage disequilibrium due to a lack of recombination or other factors such as selection, gene flow, drift, or linkage (Milgroom 1996). Maynard Smith et al. (2000) indicated that disequilibrium may occur because recombination is rare or absent or because there is geographical structuring within the populations. The New York isolates were analyzed as one population due to the lack of population structure, because there was little structure or clustering by host plant, year of collection, or county from which the isolate was collected. Lack of population structure might be a result of multiple introductions through seedborne inoculum (Iacomi-Vasilescu et al. 2002, 2004). Most of the variation observed was among the individuals.

Similar studies have also seen high levels of diversity for *A. brassicicola* populations; however, the populations studied were also found to be in significant linkage disequilibrium, indicating that clonal reproduction still exists among the populations. (Bock et al. 2005; Linde et al. 2010). Bock et al. (2005) suggested that the high levels of diversity observed were due to recombination but suggested that the populations remained in linkage disequilibrium due to the fact that recombination happens only on occasion or, perhaps, numbers have not built up for the population to be in linkage equilibrium. Linde et al. (2010) also found high levels of genetic diversity and found that all but two of the populations were in linkage equilibrium.

Other *Alternaria* spp. in addition to *A. brassicicola* have also shown indication of recombination or high diversity (Gannibal et al. 2007; Morris et al. 2000; Prasad et al. 2009; van der Waals et al. 2004). Distinct subpopulations of *A. alternata* sensu lato on citrus were found to be in linkage disequilibrium; however, when one of the subpopulations was examined further by dividing into two groups, one of the groups was found to be in linkage equilibrium even though only one mating type existed (Stewart et al. 2013). The authors suggested that a parasexual life cycle was occurring in this population.

The high levels of genotypic diversity seen in *A. brassicicola* isolates from New York State could be indicative of sexual

reproduction; however, no sexual stage has been reported and, even though the population does not appear to be clonal, this does not mean that sexual reproduction is occurring in New York State. Future studies that include a larger sample size and sampling by region may help detect whether there is any geographical structuring within populations.

There is no evidence for azoxystrobin resistance in New York at this time because we did not identify mutations at locations F129L, G137R, and G143A; however, a range of sensitivities was observed for *A. brassicicola* during in vitro assays. This is the first study that investigated the sensitivity of *A. brassicicola* to azoxystrobin, and the EC_{50} values can serve as a baseline for *A. brassicicola* to azoxystrobin. The range of EC_{50} values in *A. brassicicola* was higher than what is typically observed for baseline sensitivities in other *Alternaria* spp. In carrot, baseline sensitivities for *A. dauci* to azoxystrobin were much lower and ranged from 0.01 to 0.37 $\mu\text{g/ml}$ (Rogers and Stevenson 2010). Sensitive *Alternaria* isolates from pistachio had EC_{50} values of 0.008 to 0.045 $\mu\text{g/ml}$, whereas the resistant isolates had EC_{50} values greater than 100 $\mu\text{g/ml}$ and were found to contain the G143A mutation in the *cytb* gene (Ma et al. 2003). Pasche et al. (2005) reported that *A. solani* isolates sensitive to azoxystrobin had a mean EC_{50} value of 0.0324 $\mu\text{g/ml}$, whereas isolates containing the F129L mutation had a mean EC_{50} value of 0.3788 $\mu\text{g/ml}$. Because no mutations were found and the EC_{50} values were relatively low, we believe that no resistance has been detected at this time. Future studies are needed to assess the field efficacy of azoxystrobin against *A. brassicicola*.

In addition to determining the baseline sensitivity of *A. brassicicola* to azoxystrobin, this study also identified a large intron in the *cytb* gene after codon 131. Other studies have shown that the presence or lack of introns can have an effect on the presence of point mutations known to confer resistance to QoI fungicides (Grasso et al. 2006a). In other pathosystems, introns located after codon 143 appear to prevent the G143A mutation from developing (Fernández-Ortuño et al. 2008; Grasso et al. 2006b; Jiang et al. 2009; Samuel et al. 2011). Leiminger et al. (2014) found two genotypes for the *cytb* gene in *A. solani* isolates in Germany, with genotype I containing introns after codon 126 and 143 and genotype II with an intron after codon 131. They found that the genotype II isolates containing the intron after codon 131 also had the mutation for F129L. Because no *cytb* gene mutations were identified in the New York isolates tested, it is not known whether the presence of the intron has an effect on the presence of mutations known to confer resistance.

Isolates of *A. brassicicola* from New York were found to be highly diverse, potentially indicating recombination; however, linkage disequilibrium indicated that asexual reproduction appears to be important for the population. The population was sensitive to azoxystrobin at this time but later studies with larger populations need to be conducted to further clarify the diversity and azoxystrobin sensitivity of this pathogen in New York.

Table 4. Population size, number of multilocus genotypes (MLG), genotypic diversity, and index of association (I_A) as determined for New York State isolates of *Alternaria brassicicola* with 10 microsatellite loci

Isolates	N^a	MLG ^b	N_1^c	G^d	$1 - \lambda^e$	H_{exp}^f	E_5^g	I_A^h	P value ⁱ
<i>A. brassicicola</i>	49	44	42.52	40.7	0.975	0.53	0.956	0.143	0.028

^a Total number of isolates used in this study.

^b Number of MLG identified from the alleles at 10 microsatellite loci.

^c Proportional Shannon-Wiener's index of genetic diversity (H'). $N_1 = e^{H'}$ (Grünwald et al. 2003), $H' = -\sum_i (p_i \ln p_i)$, where p_i is the frequency of the i th MLG (Shannon 2001); statistic can have a minimum value of 1 and maximum value of the population size (N).

^d Stoddart and Taylor's index of MLG diversity (G) = $1/\sum p_i^2$, where p_i is the frequency of the i th genotype in the population (Stoddart and Taylor 1988); statistic can have a minimum value of 1 and maximum value of the population size (N).

^e Simpson's index of diversity (λ) = $\sum p_i^2$, where p_i is the frequency of the i th genotype in the population (Simpson 1949); statistic can have a value from 0 to 1.

^f Mean Nei's index of gene diversity (Nei 1978) calculated after removing nonpolymorphic loci (H_{exp}) = $1 - \sum p_i^2$, where p_i is the frequency of the i th allele at the given locus; statistic can have a value from 0 to 1.

^g Evenness (E_5) = $(G - 1)/(N_1 - 1)$ (Grünwald et al. 2003); statistic can have a value from 0 to 1.

^h Index of association (I_A) = $(V_O/V_E) - 1$, where V_O is the observed variance of differences between individuals and V_E is the expected variance (Brown et al. 1980).

ⁱ P value for I_A calculated using *poppr* (Kamvar et al. 2014). Significance was tested with 999 permutations.

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