Diversity and Virulence of *Alternaria* spp. Causing Potato Early Blight and Brown Spot in Wisconsin

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ABSTRACT

Early blight, caused by *Alternaria solani*, along with brown spot, caused by *A. alternata*, have the potential to reduce quality and yield in potato production globally. Prior to this study, the incidence, disease impact, and fungicide resistance attributes of *A. alternata* in Wisconsin were poorly understood. Potato pathogens were isolated from foliar lesions at three commercial locations in Wisconsin in 2012 and 2017 and were initially morphologically identified as *A. solani* ($n = 33$) and *A. alternata* ($n = 40$). Identifications were further corroborated with the phylogenetic analysis of the internal transcribed spacer (ITS), translation elongation factor 1 (TEF1), *gapdh*, Alt a 1, and OPA10-2. A multigene phylogeny of ITS, TEF1, *gapdh*, and Alt a 1 showed five genotypes of *A. alternata* and one single genotype of *A. solani*. We demonstrated that the *A. alternata* isolates were virulent on potato cultivars Russet Burbank ($P < 0.013$) and Atlantic ($P < 0.0073$), though they caused less disease than *A. solani* ($P < 0.0001$ and $P < 0.0001$, respectively). *A. alternata*

caused little disease on the breeding line 24-24-12 ($P = 0.9929$), and *A. solani* caused fewer disease symptoms on 24-24-12 than on Russet Burbank ($P < 0.0001$) or Atlantic ($P < 0.0001$). Breeding line 24-24-12 may be a promising source of potential resistance for the two diseases. There was no significant difference in virulence of different *A. alternata* genotypes, and no significant difference in virulence or genotype clustering among isolates from the three locations. Isolates of *A. alternata* that induced chlorosis caused larger lesion areas than isolates that did not in Russet Burbank ($P < 0.0001$), Atlantic ($P < 0.0001$), and 24-24-12 ($P = 0.0365$). There was no significant difference in virulence between quinone outside inhibitor (QoI)-sensitive and QoI-resistant isolates of *A. alternata*. This study enhanced our understanding of potato early blight and brown spot in Wisconsin, and suggested that *A. alternata* in addition to *A. solani* should be carefully monitored and possibly uniquely managed in order to achieve overall disease control.

Early blight, caused by *Alternaria solani*, is a worldwide disease problem in potato production and has the potential to reduce quality and yield up to 30% annually (Shtienberg et al. 1990). The disease is readily recognized by its distinctive bull's-eye-shaped lesions (approximately 3 to 12 mm in diameter) (Stevenson et al. 2001). A second potato disease, brown spot, caused by *A. alternata*, has been increasingly reported by commercial potato growers in Wisconsin and other regions (Fairchild et al. 2013; Soleimani and Kirk 2012; Thomma 2003; Zheng et al. 2015). Brown spot has been considered a relatively minor disease on potato because it is characterized by smaller lesions (approximately pinpoint to 10 mm in diameter) and its impact on crop health has generally been less than that of early blight (Stevenson et al. 2001). However, brown spot can result in up to 18% reduction in tuber yield at 28% foliar disease incidence when inoculum is abundant, a cultivar is highly susceptible, and environmental conditions are favorable (Droby et al. 1984). Unlike early blight, brown spot has been generally neglected in studies and is poorly understood. The two *Alternaria* pathogens share many similarities in that both are asexually propagated and cause polycyclic diseases, with initial conidial inoculum dispersed by wind and rain splash. Furthermore, both cause necrotic lesions on potato leaves and on potato tubers, albeit infrequently. The two diseases often co-occur, and the pathogens have been isolated from the same plant or even the same leaf. Although these two *Alternaria*

spp. have been considered the primary species involved in potato disease in Wisconsin, additional species have been reported from other production regions, and the diversity within our own region may change over time (Landschoot et al. 2017; Rodrigues et al. 2010).

Currently, no commercial potato cultivar is resistant to early blight or brown spot; therefore, management primarily relies upon regular fungicide applications. Most current fungicide programs target *A. solani* with the additional benefit of also managing *A. alternata* because both pathogens are closely related and have similar life cycles (Agrios 2005). Due to the polycyclic attributes of the diseases, most growers apply fungicides on a weekly basis, which results in a crop input cost of approximately \$150 per acre annually (Jordan et al. 2014). The single-site mode-of-action fungicide azoxystrobin, a quinone outside inhibitor (QoI), was often used in managing the diseases. It offered good disease control of *A. solani* when first introduced to the fields in the late 1990s in Wisconsin. However, the early blight pathogen populations developed significant resistance within 3 years of application (Rosenzweig et al. 2008a,b). The two pathogens show different responses to QoIs, which inhibit mitochondrial respiration by blocking electron transport between cytochrome *b* and cytochrome *c1* (Grasso et al. 2006). Amino acid substitutions caused by single-nucleotide mutations at amino acid positions 129 (F129L), 137 (G137R), and 143 (G143A) of cytochrome *b* confer fungicide resistance in *Alternaria* spp. (Grasso et al. 2006). In *A. solani*, F129L is more common, which leads to a partial resistance to QoIs (Pasche and Gudmestad 2008; Rosenzweig et al. 2008b), whereas, in *A. alternata*, G143A is more common, leading to a complete resistance to QoIs (Fan et al. 2015; Karaoglanidis et al. 2011; Vega et al. 2012). Pasche and Gudmestad (2008) showed that *A. solani* with an F129L mutation resulted in a lower germination rate and higher virulence on potato than the wild type. *A. alternata* with G143A has been shown to be associated with higher virulence in

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pistachio and some citrus cultivars (Karaoglanidis et al. 2011; Vega and Dewdney 2014). However, the impact of having QoI resistance in *A. alternata* during potato infection is understudied (Karaoglanidis et al. 2011; Pasche and Gudmestad 2008). This information will be crucial in order to design targeted and prescriptive management of each pathogen to achieve better control of the disease complex.

Accurate identification of plant pathogens is critical for understanding epidemiology and management methods (Meng et al. 2015a). Several *Alternaria* spp. in addition to *A. solani* have been reported to be associated with potato leaf blight, such as *A. alternata*, *A. arborescens*, and *A. tenuissima* in the U.S. northwest (Tymon et al. 2016b); *A. alternata* and *A. tenuissima* in China (Zheng et al. 2015); and *A. tomatophila* and *A. grandis* in Brazil (Rodrigues et al. 2010). It is clear that *A. solani* is the causal pathogen of potato early blight; however, whether *A. alternata* is the sole causal pathogen of brown spot is ambiguous and has not been addressed in Wisconsin. This is partly due to the complicated taxonomic classification of the *Alternaria* genus, especially for the group of small-conidiated *Alternaria* spp. to which *A. alternata* belongs (Shoaib et al. 2014; Tymon et al. 2016b; Zheng et al. 2015). Due to the similarity of conidial morphology and genetics, a number of small-conidiated *Alternaria* spp. are classified in *Alternaria* sect. *Alternaria* (Woudenberg et al. 2015a). Woudenberg et al. (2015a) examined 168 small-conidiated *Alternaria* isolates and identified 11 phylogenetic species and one species complex in *Alternaria* sect. *Alternaria*, which provided a clear and stable species classification in this section. They concluded that the identification of *Alternaria* pathogens required multiple gene loci sequencing (Woudenberg et al. 2015a). Therefore, the classification and pathogenicity of the small-conidiated *A. alternata* causing potato leaf spot warrants renewed investigation.

The goal of this work was to identify and characterize the pathogens causing early blight and brown spot in Wisconsin commercial fields and establish the pathogenicity and virulence of *A. alternata* on potato. We identified the *Alternaria* pathogens by examining five genetic regions (internal transcribed spacer [ITS], translation elongation factor 1 [TEF1], *gapdh*, Alt a 1, and OPA10-2) of 73 isolates. Furthermore, we investigated the pathogenicity of *A. alternata* on potato using three potato genotypes and explored additional pathogen characteristics such as QoI resistance and induction of chlorosis, which may influence pathogenicity.

MATERIALS AND METHODS

Sample collection. Compound leaves of potato plants exhibiting early blight or brown spot symptoms were collected from

individual commercial potato fields from three locations on 9 August 2012 and again on 25 June 2017 for genotype comparison. The locations sampled included Hancock, Plover, and Grand Marsh, WI. These three locations are at least 30 km away from each other, and the fields sampled in 2012 and 2017 from each location were different fields but within a distance of 5 km. Potato cultivar Russet Burbank was planted in all of the fields sampled in Hancock and Grand Marsh, and potato cultivar Gold Rush was planted in both of the fields sampled in Plover.

Isolation and culturing. For each sample, lesion margins were excised from each leaf, surface sterilized with 70% ethanol, and incubated on antibiotic-amended water agar (with 0.25% ampicillin and 0.05% rifampicin) to isolate the pathogens. Multiple lesions from each leaf were excised and cultured on individual Petri dishes but only one single conidium from all resulting colonies with the same conidial morphology was picked and subcultured onto clarified antibiotic-amended V8 juice agar (with 0.125% ampicillin and 0.05% rifampicin). To encourage sporulation, all cultures were incubated under 20 h of light and 4 h of darkness at 21 °C for 2 weeks in an incubator. Based on relative conidial morphology, isolates producing long conidia (70 to 90 µm) with long beaks (30 to 40 µm) were putatively identified as *A. solani*. Isolates producing small conidia (25 to 35 µm) with short beaks (4 to 6 µm) were putatively identified as *A. alternata*. In total, 40 isolates of *A. alternata* and 33 isolates of *A. solani* were obtained and used for the subsequent experiments (Table 1).

Polymerase chain reaction and sequencing. Genomic DNA extraction was performed on the isolates obtained following the protocol of Bok and Keller (2012). Five genetic regions were amplified. The ITS region was amplified with primers ITS1 and ITS4 (White et al. 1990), the TEF1 gene with EF1-728F and EF1-986R (Carbone and Kohn 1999), the Alt a 1 region with Alt-for and Alt-rev (Hong et al. 2005), the *gapdh* region with *gpd1* and *gpd2* (Berbee et al. 1999), and the OPA10-2 region with OPA 10-2R and OPA 10-2L (Andrew et al. 2009) (Table 2). These genetic regions were amplified following previously published protocols (Woudenberg et al. 2013, 2014, 2015a). Polymerase chain reaction (PCR) products were purified with a GenElute PCR Clean-Up Kit (Sigma-Aldrich) and submitted for sequencing at the Biotechnology Center, University of Wisconsin-Madison and ProteinCT Biotech. Sequences were deposited in GenBank and the accession numbers are MF279554 to MF279811 and MG525443 to MG525516.

In addition to the above genetic regions, the cytochrome *b* gene (partial) was also sequenced for the isolates to identify mutations that could influence QoI resistance. To assess mutation sites in cytochrome *b* in *A. alternata*, PCR was performed with primers DTRcytb2 (Grasso et al. 2006) and AacytbR at the annealing

TABLE 1. Location of source of *Alternaria* isolates utilized in this study

Isolates	Species	Wisconsin location of field sample collection	Year of collection
Aa35, Aa36, Aa37, Aa40, Aa43, Aa45, Aa47, Aa49, Aa52, Aa54	<i>Alternaria alternata</i>	Plover	2012
Aa55, Aa57, Aa58, Aa60, Aa62, Aa63, Aa64, Aa66, Aa67, Aa69	<i>A. alternata</i>	Hancock	2012
Aa70, Aa71, Aa72, Aa73, Aa74, Aa75, Aa76, Aa77, Aa78, Aa79	<i>A. alternata</i>	Grand Marsh	2012
SAa1, SAa2, SAa5	<i>A. alternata</i>	Grand Marsh	2017
SAa3, SAa4, SAa6, SAa8	<i>A. alternata</i>	Hancock	2017
SAa7, SAa9, SAa10	<i>A. alternata</i>	Plover	2017
As35, As38, As39, As41, As44, As45, As49, As53, As55, As57	<i>A. solani</i>	Plover	2012
As58, As59, As61, As62, As65, As69, As71, As72, As73, As76	<i>A. solani</i>	Hancock	2012
As78, As79, As80, As81, As82, As83, As84	<i>A. solani</i>	Grand Marsh	2012
SAs11, SAs12, SAs16	<i>A. solani</i>	Hancock	2017
SAs13, SAs14	<i>A. solani</i>	Plover	2017
SAs15	<i>A. solani</i>	Grand Marsh	2017

temperature of 52°C (Table 2). AacytbR was designed based on the sequence of the GenBank accessions JQ446323 and JQ437357 (Vega et al. 2012). To assess mutation sites in cytochrome *b* in *A. solani*, PCR was performed with primers As5F (Grasso et al. 2006) and Ascytb129_R at the annealing temperature of 50°C for F129L, and Ascytb143_F and Ascytb143_R at the annealing temperature of 59°C for G137R and G143A. The latter three primers were designed based on the sequence of the GenBank accession DQ209284 (Grasso et al. 2006). The amplified segments were sequenced and aligned to detect the single-nucleotide polymorphisms (SNPs) potentially causing the F129L, G137R, and G143A substitutions.

Phylogenetic analysis. Sequences of each genetic region were aligned with reference sequences from GenBank (Supplementary Table S1) using ClustalW in BioEdit v7.0.5.3 (Hall 1999) with manual adjustment. Phylogenetic analysis was conducted with each of the five genetic regions separately; then, subsequent phylogenetic analyses were conducted with combined genetic regions of ITS, TEF1, Alt a 1, and *gapdh*. Phylogenetic trees with each genetic region and the combined dataset were generated with maximum-likelihood (ML) analysis, including 1,000 bootstrap replicates using ATGC PhyML 3.0 (<http://www.atgc-montpellier.fr/phyml/>) (Guindon et al. 2010). The best model for the ML analysis was determined with ATGC Smart Model Selection (<http://www.atgc-montpellier.fr/phyml-sm/>). Additionally, a Bayesian analysis was performed on each genetic region and the combined aligned dataset with BEAST v1.5.4 (Drummond et al. 2012). For calculating the Bayesian posterior probabilities, the Markov Chain Monte Carlo chain was set to 1,000,000, sample frequency was set at 100 for each analysis, and burn-in was set to 2,000. Finally, the phylogeny for each genetic region and multigene phylogeny were displayed with the phylogenetic tree generated using the ML method but both bootstrap values and Bayesian posterior probabilities (pp values) were indicated at the nodes of the tree. We used the SH test (Shimodaira and Hasegawa 1999), as implemented in IQ-TREE v1.6.7.a (Nguyen et al. 2015), to evaluate whether each ML topology computed with separate regions was significantly worse than the best ML tree obtained from the combined dataset. The test was conducted using 10,000 bootstrap replicates.

Plant materials. Three potato genotypes (cultivars Russet Burbank and Atlantic and breeding line 24-24-12) were used in this study to ensure a comprehensive virulence test (Geuens et al. 2008; Nie et al. 2012). Russet Burbank and Atlantic are widely grown commercially, and are susceptible to early blight (Weber and Halterman 2012); breeding line 24-24-12 was selected for robust early blight resistance and genetic self-compatibility (Halterman et al. 2015). The three aforementioned potato genotypes exhibit different levels of susceptibility to early blight caused by *A. solani*

(Weber and Halterman 2012) but susceptibility to *A. alternata* had previously not been evaluated.

Virulence test. Russet Burbank, Atlantic, and breeding line 24-24-12 were grown in a disease-free greenhouse (22°C [day] and 18°C [night], with 16-h photoperiod) at the University of Wisconsin-Madison Walnut Street Greenhouse facility for 2 months prior to establishment of the virulence test. Compound leaves were collected from the plants by cutting the petiole of the leaf at the third and fourth node from the top of the plant with a sterile razor blade. Four detached potato leaves from each potato genotype were placed on a wire mesh with petiole pointing downward in a translucent plastic box (with the lid closed but not sealed), producing a high-humidity environment, as one inoculation system, and all of the boxes were placed in a greenhouse maintained at 22°C during the day and 18°C at night with a 16-h photoperiod (Fig. 1). Each box contained 2-cm-deep water and a wire mesh to support leaves in an upright position. Petioles were submerged in the water to maintain plant tissue hydration and turgor. Conidia of the 30 2012 *A. alternata* isolates were collected by flooding the 2-week-old culture plates with sterile water (containing 1% Tween 20); the concentration of the conidia suspensions was adjusted to 10⁵ conidia/ml. The inoculation was conducted by spraying the conidia suspension on the adaxial surface of the detached leaves in the translucent plastic boxes. Water was used for mock (control) inoculation and three *A. solani* isolates (As49, As72, and As84) were used as the positive control group (Table 1). The three *A. solani* isolates were cultured and had conidia collected in the manner previously described for *A. alternata*, and conidia suspensions were adjusted to 10⁴ conidia/ml. The experiment was performed three times as a factorial arrangement in a randomized complete block design, where each repetition contained 34 treatments: 33 *Alternaria* isolates and one mock inoculation with water. For each inoculation, the leaves were scanned 7 days postinoculation using an Epson V700 desktop scanner at 200 dpi.

The images of inoculated leaves were processed with ImageJ (Rasband, W.S.; ImageJ, U.S. National Institutes of Health). Both lesion area and total leaf area were selected manually by adjusting the color thresholds to hue (0, 50 to 60), saturation (0, 255), and brightness (0, 60 to 70). Disease severity was determined by calculating the percentage of lesion area from the total leaf area. Leaf yellowing or chlorotic halo phenotypes were also recorded as 0 = no chlorosis and 1 = chlorosis.

Statistical analysis. The relative abundance of each genotype (identified based on multigene phylogeny of ITS, TEF1, Alt a 1, and *gapdh*) from 2012 and 2017 were compared using the PAIRED statement with the PROC TTEST procedure in SAS (v.9.4; SAS Institute Inc.).

TABLE 2. Primer pairs used to amplify and sequence genetic regions for *Alternaria* spp. genotyping^z

Genetic regions	Primers	Sequences (5' to 3')	References
ITS	ITS1	TCCGTAGGTGAACCTGCGG	White et al. 1990
	ITS4	TCCTCCGCTTATTGATATGC	White et al. 1990
TEF1	EF1-728F	CATCGAGAAGTTCGAGAAGG	Carbone and Kohn 1999
	EF1-986R	TACTTGAAGGAACCTTACC	Carbone and Kohn 1999
Alt a 1	Alt-for	ATGCAGTTCACCACCATCGC	Hong et al. 2005
	Alt-rev	ACGAGGGTGAYGTAGGCGTC	Hong et al. 2005
OPA10-2	OPA 10-2R	GATTTCGAGCAGGGAACTA	Andrew et al. 2009
	OPA 10-2L	TGCGAGTAAGACACATTCTACG	Andrew et al. 2009
<i>gapdh</i>	gpd1	CAACGGCTTCGGTCGCATTG	Berbee et al. 1999
	gpd2	GCCAAGCAGTTGGTTGTGC	Berbee et al. 1999
Cyt <i>b</i> (partial)	DTRcytb2	CTAGTATGAACCTATTGGTAC	Grasso et al. 2006
	AacytbR	TGTAACCGTCTCCGTCTATCAATC	...
Cyt <i>b</i> (partial)	As5F	AGAACTCTAGTATGAACCTATTGG	Grasso et al. 2006
	Ascytb129_R	CATAAAATCTACCAGTTGAGGC	...
Cyt <i>b</i> (partial)	Ascytb143_F	GATTAAAGTGGTATAGCCCCGA	...
	Ascytb143_R	GATCTGTGCCTTTTGGCGTATCATCTG	...

^z ITS = internal transcribed spacer, TEF1 = translation elongation factor 1, and Cyt *b* = cytochrome *b*.

The change in relative abundance of *Alternaria* spp. isolates with QoI resistance from 2012 to 2017 was analyzed with a χ^2 test. The expected number of resistant isolates in 2017 was derived from their relative abundance in 2012 and then compared with the observed numbers in 2017. The difference between expected values and observed values was determined at the $\alpha = 0.05$ significance level.

Disease severity was compared among inoculations with *A. alternata*, *A. solani*, and water, and the differences among the three groups were determined by Tukey's test at the $\alpha = 0.05$ significance level using the LSMeans statement of PROC GLM analysis in SAS. The influence of genotype, location, chlorosis, and QoI resistance mutation on disease severity was statistically analyzed the same way using PROC GLM analysis in SAS for the 30 isolates of *A. alternata* from 2012.

RESULTS

Phylogenetic analysis. Phylogenetic analysis supported our morphological identification of *A. solani* and *A. alternata* isolates derived from symptomatic potato leaves.

There was no sequence diversity in *A. solani*, as indicated by no SNPs. Both single-gene (ITS [Supplementary Fig. S1], *gapdh* [Supplementary Fig. S2], TEF1 [Supplementary Fig. S3], and Alt a 1 [Fig. 2]) and multigene (Fig. 3) phylogeny distinguished *A. solani* from other species and showed only one genotype.

A. alternata showed sequence diversity. Specifically, 1, 3, 1, and 24 SNPs were observed in ITS (455 aligned characters), *gapdh* (579 aligned characters), TEF1 (240 aligned characters), and Alt a 1 (472 aligned characters) genes, respectively, in the *A. alternata* isolates. With the *A. alternata*-specific OPA10-2 gene (634 aligned characters), 31 SNPs were observed.

Single-gene phylogenies with sequences of ITS, *gapdh*, and TEF1 could not distinguish different species in the small-conidiated *Alternaria* group known as sect. *Alternaria* (Woudenberg et al. 2015a). However, both Alt a 1 and OPA10-2 distinguished most species in sect. *Alternaria*, and separated *A. alternata* into three and six groups, respectively (Figs. 2 and 4). Though the morphologically identified *A. alternata* isolates grouped differently with Alt a 1 and OPA10-2, all of them fell into the same clusters with reference isolates that were identified as *A. alternata* by previous studies (Woudenberg et al. 2015a). Combining the datasets of ITS, *gapdh*, TEF1, and Alt a 1 resulted in a significantly better ($P < 0.01$) ML topology than ITS, *gapdh*, and TEF1 individually according to the SH test, including Alt a 1 ($P = 0.0187$) individually when employing a weighted (standardized) version of the SH test. The multigene phylogeny (Fig. 3) also distinguished each *Alternaria* sp., and five genotypes were resolved. Genotype I *A. alternata* was predominant and represented 58% of the total *A. alternata* isolates. There were no clusters of *A. alternata* genotype by location across each of the three locations sampled (Hancock, Plover, or Grand Marsh) but, rather, a mixture of multiple genotypes was detected from each location. Most of the genotypes discovered in 2012 were retrieved again in 2017 with the same overall relative abundance in the population (Table 3).

QoI resistance. Cytochrome *b* (partial) sequences of *A. alternata* showed that 76.6% of the 2012 isolates and 40% of the 2017 isolates had one nucleotide mutation at amino acid position 143 from GGT to GCT, leading to a switch of amino acid from glycine to alanine; none of the isolates showed mutation at amino acid position 129 or 137. For *A. solani*, 96% of the isolates from 2012 and 100% from 2017 showed nucleotide mutations at amino acid position 129 from TTC to CTC, TTA, or TTG, all leading to a switch of amino acid from phenylalanine to leucine; none of the isolates showed mutations at amino acid position 137 or 143. The relative abundance of *A. alternata* isolates that were QoI resistant increased from 2012 to 2017 ($\chi^2 = 7.11$, $P < 0.01$), whereas it stayed the same in *A. solani* ($\chi^2 = 0.25$, $P > 0.5$) (Table 4).

Virulence test. Symptoms caused by both *Alternaria* spp. in the inoculation experiment were consistent with field observations. Potato cultivars Russet Burbank and Atlantic showed large, dark-brown lesions when inoculated with *A. solani* and small, brown lesions when inoculated with *A. alternata*. No symptoms were observed with water-treated mock inoculations (Fig. 5). Breeding line 24-24-12 showed relatively mild symptoms when inoculated with *A. solani*, and few to no symptoms when inoculated with *A. alternata* (Fig. 5). *Alternaria* spp. were reisolated consistently from all leaf lesions, including 24-24-12, confirming the cause of the symptoms.

Disease severity caused by *Alternaria* spp. ranged from approximately 1% on 24-24-12 to approximately 15% on Russet Burbank. There were statistical differences in virulence between disease responses of *A. alternata* and *A. solani* inoculation on the three potato genotypes (Russet Burbank, $P < 0.0001$; Atlantic, $P < 0.0001$; and 24-24-12, $P < 0.0001$). However, there were no statistical differences in disease between individual isolates within *A. alternata* and *A. solani* across potato genotypes (Supplementary Fig. S4). Overall, *A. solani* caused similar disease severity of roughly 15 and 14% on Russet Burbank and Atlantic, respectively. *A. solani* caused only approximately 7% severity on 24-24-12, which was significantly less than that on Russet Burbank ($P < 0.0001$) or on Atlantic ($P < 0.0001$). The same disease severity trend was observed when potato genotypes were inoculated with *A. alternata* (3, 3, and approximately 1%, respectively) (Fig. 6). Within each potato genotype, the mean disease severity caused by *A. alternata* was significantly lower than that caused by *A. solani* ($P < 0.0001$ for each) (Fig. 6). The mean disease severity caused by *A. alternata* was significantly greater than the mock inoculation on Russet Burbank ($P = 0.0130$) and Atlantic ($P = 0.0073$) but not on 24-24-12 ($P = 0.9929$).

Disease severities were also compared between *A. alternata* isolates categorized by isolate genotype, location of collection, chlorotic response, and presence of the QoI resistance mutation. Of the 30 *A. alternata* isolates tested, those that caused chlorosis also resulted in greater disease severity than isolates that did not cause chlorosis on all of Russet Burbank ($P < 0.0001$), Atlantic ($P < 0.0001$), and 24-24-12 ($P = 0.0365$) (Table 5). Generally, different genotypes of *A. alternata* caused similar disease severity, with the exception of genotype V, which resulted in a moderately higher disease severity on Russet Burbank. There was no significant difference in disease caused by isolates from the three locations on Atlantic or 24-24-12; however, *A. alternata* from Plover caused significantly less disease on Russet Burbank (Table 5). No

Fig. 1. Inoculation system for each *Alternaria* isolate. Example of the inoculation chambers established in a greenhouse to inoculate *Alternaria* isolates onto detached potato compound leaves.

significant difference in disease severity between QoI fungicide-resistant or -sensitive isolates was detected.

DISCUSSION

Through both phenotypic and genotypic analyses, we identified and characterized *A. solani* and *A. alternata* as the pathogens causing early blight and brown spot, respectively, in Wisconsin commercial fields in 2012 and 2017. We compared virulence,

chlorotic lesion phenotype, and QoI resistance of potato-pathogenic *A. alternata* with *A. solani* co-occurring in field populations from central Wisconsin. We also assessed and compared *A. solani* and *A. alternata* virulence on a selected group of potato genotypes to improve understanding of disease response in common cultivars and an experimental potato breeding line.

Based on the multigene phylogenetic analyses (ITS, TEF1, *gapdh*, and Alt a 1), we confirmed the putative, morphology-based species identification of the pathogens as *A. solani* and *A. alternata*.

Fig. 2. Phylogenetic tree constructed based on the Alt a 1 gene of the *Alternaria* isolates and reference sequences retrieved from GenBank (accession numbers shown in tree). The tree was constructed by maximum-likelihood, and bootstrap values (1,000 replications) are shown in front of the slash at each node. Bayesian posterior probabilities (%) were also calculated and the values are shown after the slash. Only bootstrap values > 500 and Bayesian pp > 70% are shown. An asterisk (*) indicates a Bayesian pp value < 70%. Samples without underlines were collected from 2012 and underlined samples were collected from 2017; "All *A. solani*" means all 33 isolates of *Alternaria solani* collected from both 2012 and 2017.

Genetic regions such as ITS, TEF1, and *gapdh* are conserved and are often used to identify fungal species (Damm et al. 2012; Phillips et al. 2013; Samson et al. 2014; Woudenberg et al. 2013). Another gene, Alt a 1, which codes for an *Alternaria* allergen and is associated with asthma during human exposure (Chruszcz et al. 2012), was also used in combination with ITS, TEF1, and *gapdh* in identifying the *Alternaria* spp. The multigene phylogeny with the combined dataset showed an ML topology that is significantly better than all alternatives derived from a single region in identifying small-conidiated *Alternaria* spp. Therefore, the multi-gene phylogeny was used to identify different genotypes of *A. alternata* in this study. However, both the phylogenetic analysis of each genetic region and multiple genes combined indicated that Alt a 1 alone or multigene phylogeny showed high resolution. This suggests that Alt a 1, which evolved rapidly but had a well-preserved secondary structure in most species of *Alternaria* (Hong et al. 2005), is a good molecular marker to use in identifying *A. alternata*.

Another gene, OPA10-2, which codes for a protein with unknown function, was not used in the multiple gene phylogenetic analysis for species identification because it was only amplified in the small-conidiated *Alternaria* spp. Though the topology of the ML trees constructed with OPA10-2 was different from that with Alt a 1 or the multigene phylogeny, all morphologically identified *A. alternata* isolates fell into the cluster with *Alternaria* spp. previously identified as *A. alternata* (Woudenberg et al. 2015a). This indicated that all of these small-conidiated isolates collected in this study could be classified as *A. alternata* but there was high genotypic diversity within them, as was found in Woudenberg et al. (2015b).

Despite the fact that multiple *Alternaria* spp. have been reported to be associated with potato leaf blight in other regions, we have only identified *A. solani* and *A. alternata* thus far in Wisconsin. Isolates of both species used in this study were isolated from symptomatic potato leaves, suggesting that both species were adapted to potato. Our virulence test results showed that *A. solani* was highly infectious on potato and caused more disease than

Fig. 3. Phylogenetic tree constructed based on the Alt a 1, internal transcribed spacer (ITS), translation elongation factor 1 (TEF1), and *gapdh* genes of the *Alternaria* isolates and reference sequences retrieved from GenBank (accession numbers shown in tree). The tree was constructed by maximum-likelihood and bootstrap values are shown in front of the slash. Bayesian posterior probabilities (%) were also calculated and the values are shown after the slash at each node. Only bootstrap values > 500 and Bayesian pp value > 70% are shown. Samples without underlines were collected from 2012 and underlined samples were collected from 2017; "All *A. solani*" means all 33 isolates of *Alternaria solani* collected from both 2012 and 2017.

A. alternata on all the three potato genotypes tested. This finding is consistent with comparative *Alternaria* spp. reports on potato from other locations (Stammler et al. 2014; Tymon et al. 2016a). It is possible that *A. solani* has had a longer period of adaption to potato compared with *A. alternata* and, therefore, caused more severe disease. *A. alternata* has a broad host range and may have only recently migrated from another host and adapted to potato, as indicated by reports that were only initiated during the 1980s (Droby et al. 1984).

Though less aggressive than *A. solani* on potato, variation in virulence was observed among the *A. alternata* isolates. Disease severity caused by different genotypes of *A. alternata* did not differ, indicating that genotypic diversity based on the genetic regions studied in this work did not contribute to the virulence or fitness of the pathogen. This may explain the consistency of relative abundance of different genotypes in *A. alternata* populations collected from infected potato plants over time from 2012 to 2017 (Table 3). However, lesion area was greater when the isolates caused chlorosis. Chlorotic halos are indications of the presence of phytotoxin (Ayer and Penarodriguez 1987), which may be responsible for the higher virulence of chlorosis-inducing *A. alternata* isolates.

Our findings support the idea of genetic mixing because there was no difference in virulence of the *A. alternata* isolates from different geographical regions and no distribution pattern of different genotypes observed in this study. Both *Alternaria* spp. propagate asexually and produce numerous conidia that are dispersed by wind and rain splash. Populations can be mixed easily, and genotypes

may get mixed through asexual recombination such as hyphal fusion or anastomosis, a possible mechanism of intraspecies horizontal chromosome transfer commonly observed and even necessary for pathogenicity in *Alternaria* spp. (Craven et al. 2008; van der Waals et al. 2004). In addition to the shared source of diversity, cryptic sex, described by Meng et al. (2015a), may also be a mechanism for *A. alternata* to exchange genetic information and provides an explanation for the high intraspecies diversity of *A. alternata*. Although the one genotype based on the genes analyzed in this study does not necessarily imply a clonal *A. solani* population, other gene markers or an alternative research approach

TABLE 3. Comparison of the relative abundance of different genotypes in *Alternaria alternata* population from 2012 and 2017^z

Genotype	Relative abundance in 2012 (%)	Relative abundance in 2017 (%)
I	56.67	60.00
II	10.00	20.00
III	16.67	10.00
IV	6.67	0.00
V	10.00	10.00
Total number of isolates	30	10
Statistics	$t = 0.0006, P = 0.9995$	

^z Genotypes were categorized based on the groupings from multigene phylogeny of the internal transcribed spacer (ITS), translation elongation factor 1 (TEF1), *gapdh*, and *Alt a 1*.

Fig. 4. Phylogenetic tree constructed based on the OPA10-2 gene of the *Alternaria alternata* isolates and reference sequences retrieved from GenBank (accession numbers shown in tree). The tree was constructed by maximum-likelihood, and bootstrap values (1,000 replications) are shown in front of the slash at each node. Bayesian posterior probabilities (%) were also calculated and the values are shown after the slash. Only bootstrap values > 500 and Bayesian pp value > 70% are shown. Samples without underlines were collected from 2012 and underlined samples were collected from 2017.

may well capture its diversity (Meng et al. 2015b; van der Waals et al. 2004; Weir et al. 1998).

Given the continued use of QoI fungicides in some commercial potato production programs in Wisconsin, we wanted to better understand the sensitivity of the pathogen in representative populations within the state. We characterized a high relative abundance of QoI-resistant *A. solani* isolates both in 2012 and 2017. *A. solani*, an exclusive potato and tomato pathogen, has possibly received increased management pressure with QoI applications over the past two decades, resulting in the relatively high and consistent relative abundance of QoI-resistant isolates between the 2 years tested. However, the relative abundance of QoI-resistant *A. alternata* isolates was relatively lower than that in *A. solani*, and the relative abundance decreased from 2012 to 2017. Such a decrease was surprising, given that QoI sensitivity is unlikely to

TABLE 4. Quinone outside inhibitor (QoI) fungicide sensitivity responses of *Alternaria* spp. collected in 2012 and 2017 from commercial potato fields of Wisconsin^z

Pathogen, QoI sensitivity	Number of isolates in each year	
	2012	2017
<i>Alternaria alternata</i>		
Sensitive	7	6
Resistant	23	4
Statistics	$\chi^2 = 7.11, P < 0.01$	
<i>A. solani</i>		
Sensitive	1	0
Resistant	26	6
Statistics	$\chi^2 = 0.25, P > 0.5$	

^z QoI resistance was determined by presence of nucleotide mutation of cytochrome *b*. QoI resistance of *A. alternata* was due to the mutation of GGT to GCT on cytochrome *b* amino acid position 143. QoI resistance of *A. solani* was due to the mutation of TTC to TTA, CTC or TTG on cytochrome *b* amino acid position 129.

Fig. 5. Symptoms of infection caused by representative isolates of *Alternaria alternata* and *A. solani* inoculated onto detached compound leaves of three potato genotypes. Images were taken 7 days postinoculation.

return without selection pressure because there is no fitness cost (Karaoglanidis et al. 2011; Vega and Dewdney 2014). It is possible that the relative abundance of QoI-resistant isolates from 2012 and 2017 may not be appropriate to compare because the samples in 2012 were collected later in the season than the 2017 samples.

We believe that *A. alternata*, with a wide host range, may have a wild-type reservoir without QoI selection pressure. The QoI-sensitive conidia can then move into potato fields early in the season, leading to a low initial relative abundance of QoI-resistant isolates. As QoI fungicides are applied in fields, the resistant

Fig. 6. Virulence of *Alternaria alternata* and *A. solani* on three genotypes of potato plants. The column labeled “*Alternaria alternata*” is the mean of the disease severity of 30 *A. alternata* isolates tested; the column labeled “*Alternaria solani*” is the mean of the disease severity of 3 *A. solani* isolates tested. Error bars represent standard errors of the mean values from all data points in each category. Different letters represent significant difference based on $\alpha = 0.05$.

TABLE 5. Influence of isolate genotype, location, chlorosis, and quinone outside inhibitor (QoI) fungicide resistance of *Alternaria alternata* on disease severity on three potato genotypes (expressed in lesion percentage of total leaf area)

Potato genotype, attribute	LSMean lesion (%) ^y		
	Russet Burbank	Atlantic	24-24-12
Genotype ^z			
I	2.91 b	2.99 a	0.74 a
II	2.59 b	2.61 a	0.81 a
III	3.35 ab	3.14 a	0.92 a
IV	2.22 b	2.60 a	0.49 a
V	4.43 a	3.52 a	0.88 a
Location			
Hancock	2.90 ab	2.96 a	0.85 a
Plover	2.62 b	3.27 a	0.62 a
Grand Marsh	3.61 a	2.79 a	0.86 a
Chlorosis			
No chlorosis	1.77 a	2.49 a	0.74 a
Present	3.90 b	3.49 b	1.22 b
QoI resistance			
Resistant	3.09 a	2.99 a	0.74 a
Sensitive	2.92 a	3.04 a	0.88 a

^y Comparisons of disease severity were made within each attribute (column) for each potato genotype. Values followed by the same letter indicate no significant difference at the $\alpha = 0.05$ significance level.

^z Genotype is based on the groupings from multigene phylogeny of the internal transcribed spacer (ITS), translation elongation factor 1 (TEF1), *gapdh*, and *Alt* a 1.

population would then accumulate, resulting in an increased relative abundance of QoI-resistant isolates later in the production season. However, to validate the above assumption, QoI resistance should be profiled both early and late in the season in the same year repetitively for a reliable comparison. With a relative abundance of QoI-resistant isolates of 40 to 77% in *A. alternata*, and as high as 100% in *A. solani*, QoI fungicides may offer some brown spot control early in the season but does not seem to offer effective control to *Alternaria* diseases as the season progresses in time; therefore, alternative management strategies should be considered.

The shift of QoI sensitivity in the 2012 *A. alternata* isolates did not appear to influence virulence in *A. alternata*. G143A, the amino acid shift that confers QoI resistance in *A. alternata*, is common in a number of plant-pathogenic fungi but the influence of it in pathogen virulence varies and can be host and pathogen isolate or population specific (Hagerty and Mundt 2016; Karaoglanidis et al. 2011; Vega and Dewdney 2014). G143A in *A. alternata* was shown to associate with higher virulence when specific pathogen isolates infect certain hosts, such as *A. alternata* on pistachio and *A. alternata* on citrus (Karaoglanidis et al. 2011; Vega and Dewdney 2014). However, QoI resistance conferred by the same amino acid shifting in *Zymoseptoria tritici* and *Magnaporthe oryzae* was associated with a decrease in virulence (Hagerty and Mundt 2016; Ma and Uddin 2009), whereas it showed no association with virulence change in *M. grisea* (Avila-Adame and Koller 2003). Given the high level of genotype mixing within *A. alternata* populations discussed above, and the fact that G143A can arise independently (Hagerty and Mundt 2016), it is possible that the sole factor of QoI resistance was not significantly associated with virulence of the potato-pathogenic *A. alternata* isolates in this study.

Resistant potato cultivars would provide the best and most sustainable early blight and brown spot management. Investigations of disease resistance in experimental breeding lines are often limited to single representative isolates of plant pathogens for efficiency in screening. However, results from a multipathogen and multiisolate screen can further accelerate the advancement of disease resistance in lines destined for commercialization. Both *A. solani* and *A. alternata* exhibited reduced virulence on breeding line 24-24-12 compared with Russet Burbank and Atlantic, suggesting that 24-24-12 could be a potential breeding source for the two diseases. Line 24-24-12 is the progeny of 24-24 and M6 (*Solanum chacoense*) (Spooner et al. 2014), and 24-24 is the progeny of US-W4 (a cultivated diploid *S. tuberosum* breeding line) (Spooner et al. 2014) crossed by rap119-2 (*S. raphanifolium* 473369). Rap119-2 was found to possess a unique resistance to early blight that was not the result of late plant maturity but, rather, of minimizing lesions and reducing the capacity of *A. solani* to sporulate on the plant foliar tissues (Weber and Jansky 2012). Unfortunately, this attribute was lost through the breeding process of 24-24-12 as the pathogen sporulated and was reisolated from the inoculation sites in our experiments. Despite this, *A. solani* caused less disease on 24-24-12 than on cultivated potato and *A. alternata* did not produce any disease symptoms, indicating that 24-24-12 did inherit some resistance from *S. raphanifolium*. The combination of resistance to both *A. solani* and *A. alternata* with genetic self-compatibility made 24-24-12 an interesting and promising line for further developing commercial potato with early blight and brown spot resistance.

In summary, this study illustrated that *A. alternata* had a higher genotypic diversity, lower virulence, and lower relative abundance of QoI-resistant isolates than *A. solani*, which made *A. alternata* a different pathogen from *A. solani* and a unique pathogen in potato diseases which, therefore, should not be neglected in potato disease management strategies. High genotypic diversity and multiple methods of reproduction typically suggest a high gene flow (McDonald and Linde 2002; Meng et al. 2015a), which made the accumulation of fungicide resistance less of a concern in *A. alternata* than in *A. solani* under careful monitoring. However,

such high gene flow also means more sources of inoculum for *A. alternata*, because it indeed has a wide host range. Therefore, careful field site selection and management by avoiding sources of surrounding alternate hosts may help reduce inoculum. This study provided a better understanding of the potato *Alternaria* pathogens and may lead to a more robust and sustainable disease management strategy that does not rely solely on fungicide applications.

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