Phomopsis Stem Canker: A Reemerging Threat to Sunflower (*Helianthus annuus*) in the United States

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ABSTRACT

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Phomopsis stem canker causes yield reductions on sunflower (*Helianthus annuus* L.) on several continents, including Australia, Europe, and North America. In the United States, Phomopsis stem canker incidence has increased 16-fold in the Northern Great Plains between 2001 and 2012. Although *Diaporthe helianthi* was assumed to be the sole causal agent in the United States, a newly described species, *D. gulyae*, was found to be the primary cause of Phomopsis stem canker in Australia. To determine the identity of *Diaporthe* spp. causing Phomopsis stem canker in the Northern

Great Plains, 275 infected stems were collected between 2010 and 2012. Phylogenetic analyses of sequences of the ribosomal DNA internal transcribed spacer region, elongation factor subunit 1- α , and actin gene regions of representative isolates, in comparison with those of type specimens, confirmed two species (*D. helianthi* and *D. gulyae*) in the United States. Differences in aggressiveness between the two species were determined using the stem-wound method in the greenhouse; overall, *D. helianthi* and *D. gulyae* did not vary significantly ($P \leq 0.05$) in their aggressiveness at 10 and 14 days after inoculation. These findings indicate that both *Diaporthe* spp. have emerged as sunflower pathogens in the United States, and have implications on the management of this disease.

Additional keyword: pathogenicity.

Phomopsis stem canker is widespread in many sunflower (*Helianthus annuus* L.) regions in the world (Gulya et al. 1997; Masirevic and Gulya 1992; Thompson et al. 2011). The disease has been one of the primary limiting factors for sunflower production in Europe, where yield losses up to 50% and losses in oil content in excess of 10% have occurred (Laville 1986; Masirevic and Gulya 1992). According to data from the annual Sunflower Crop Survey coordinated by the U.S. National Sunflower Association (Mandan, ND), incidence of fields with Phomopsis stem canker has increased from approximately 1.5% of the U.S. crop in 2001 to 24.4% in 2012 (Kandel 2012). Historically, yield and oil losses due to Phomopsis stem canker in the United States have been minimal (Kandel 2012). However, in 2010, a Phomopsis stem canker epidemic occurred on sunflower in the Northern Great Plains, with incidence highest in North Dakota, South Dakota, and Minnesota, where over 75% of the U.S. sunflower crop is grown. Several isolated fields in North Dakota and Minnesota had an incidence of over 50% of the plants infected and estimated yield losses up to 40%. It was unclear what triggered the sudden reemergence of Phomopsis stem canker in 2010, given that the disease was first recorded in North Dakota and Minnesota in 1984 (Hajdu et al. 1984). In response to the epidemic, the National Sunflower Association declared the development of management tools for Phomopsis stem canker one of their top research priorities (<http://sunflowernsa.com>).

The pathogen causing Phomopsis stem canker on sunflower was first described as *Diaporthe helianthi* Munt.-Cvetk., Mihaljč. & M. Petrov (anamorph *Phomopsis helianthi* Munt.-Cvetk., Mihaljč. & M. Petrov (Muntañola-Cvetković et al. 1985) in the former Yugoslavia in 1980. Although *D. helianthi* was described as the causal agent (Aćimović and Štraser 1982; Muntañola-Cvetković et al. 1985), the possibility of multiple species infecting sunflower was raised by the researchers in the early 1980s (Muntañola-Cvetković et al. 1985). However, there was little evidence to support the multiple-species hypothesis. At that time, host association was used for identification of *Diaporthe* spp., because morphological and culture characteristics are inadequate or unreliable for species differentiation (van Rensburg et al. 2006). Consequently, all *Diaporthe* spp. isolated from infected sunflower were assumed to be caused by *D. helianthi*. However, there appeared to be distinct biological differences between *D. helianthi* isolates causing disease on sunflower in Europe and those causing disease on sunflower in the United States (Gulya et al. 1997). The U.S. isolates readily produced perithecia in culture but European isolates did not. Despite some evidence of biological differences among the *Diaporthe* isolates and a hypothesis suggesting that multiple species were involved, disease symptoms associated with the pathogen on sunflower were thought to be the same everywhere. Additionally, it was reported by Herr et al. (1983) that the severity of symptoms expressed in the commercial sunflower hybrids inoculated with the same isolate varied from plant to plant, further complicating phenotyping. The molecular evidence provided more recently strongly supports the hypothesis that multiple *Diaporthe* spp. can cause Phomopsis stem canker on sunflower (Thompson et al. 2011). Three *Diaporthe* spp. (*D. gulyae* R. G. Shivas, S. M. Thomps. & A. J. Young; *D. kongii* R. G. Shivas, S. M. Thomps. & A. J. Young; and *D. kochmanii* R. G. Shivas, S. M. Thomps. &

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A. J. Young) were responsible for the disease outbreaks in Australia in 2009 (Thompson et al. 2011), where *D. helianthi* has not been identified and remains a biosecurity threat.

When multiple pathogenic species cause the same disease, it is important to determine whether there are differences in aggressiveness among them, particularly as it relates to breeding for plant resistance. The term “aggressiveness”, as used in this article, is based on the quantitative variation of pathogenicity on susceptible hosts (Pariad et al. 2009). Variation in aggressiveness among *D. helianthi* isolates has been reported on sunflower (Encheva and Kiryakov 2002). Herr et al. (1983) examined the reaction of commercial sunflower hybrids inoculated with 15 *D. helianthi* isolates and observed variation in aggressiveness characteristics among the isolates, such as differences in lesion size and infectious period. Additionally, it was reported by Herr et al. (1983) that the severity of symptoms expressed in commercial sunflower hybrids varied from plant to plant when they were inoculated with the same *D. helianthi* isolate, complicating phenotyping. Among the *D. gulyae* isolates, variation in symptoms and lesion size that these produce was reported on sunflower by Thompson et al. (2011). Says-Lesage et al. (2002) further showed that extensive genetic differences occur among *D. helianthi* strains from different geographical origins; therefore, it is likely that there may be genetic variability between the populations of *D. helianthi* and *D. gulyae* occurring in the United States and in other sunflower production regions of the world, making evaluation of local populations critical for disease management. Although *D. helianthi* has caused significant yield losses in Europe (Masirevic and Gulya 1992) and *D. gulyae* has caused severe Phomopsis stem canker epidemics in Australia (Thompson et al. 2011), a direct comparison of aggressiveness of *D. gulyae* and *D. helianthi* has not been done.

The specific objectives of this study were to (i) phylogenetically characterize isolates of *Diaporthe* from sunflower causing Phomopsis stem canker in the Northern Great Plains and (ii) examine the aggressiveness of the *Diaporthe* isolates from sunflower. Among the major findings of this study, we have determined that at least two distinct *Diaporthe* spp. have emerged as sunflower pathogens in the United States.

MATERIALS AND METHODS

Survey collection and identification of *Diaporthe* isolates. A survey of sunflower fields was conducted in the Northern Great Plains states of Minnesota, North Dakota, and South Dakota from 2010 to 2012 in an effort to identify sunflower stem pathogens. In 2010, 55 sunflower fields were selected arbitrarily, with no fields closer than 5 km from a previously selected field. In each field, one to two 10-m-long transects (rows) were randomly selected for survey and collection. Two to three sunflower stalks exhibiting a variety of stem symptoms (wilting, lodging, and lesions of varying size and color) were arbitrarily collected in each transect.

In 2011 and 2012, the survey and collection of stem pathogens specifically targeted Phomopsis stem canker. In all, 51 and 26 fields with Phomopsis stem canker were arbitrarily selected in 2011 and 2012, respectively, and two to three diseased stalks (Supplementary Figs. 1 and 2) were collected along one to two 10-m-long transects in each field. In total, 234 isolates (83 isolations in 2010, 96 in 2011, and 55 in 2012) were recovered from 275 stalks collected (Table 1).

Stem samples were washed with tap water for 2 min, and 1-cm-long pieces were cut from infected tissue. The pieces were surface sterilized in sodium hypochlorite (10%) and ethanol (70%) for 1 min each, rinsed in sterile distilled water four times, and blotted between sterile filter papers. Four pieces were placed on potato dextrose agar (PDA; Difco Laboratories, Detroit) amended with 0.02% streptomycin sulfate (Sigma-Aldrich, St. Louis) to avoid bacterial growth. Plates were incubated at room temperature for 7 to 14 days under alternating light and dark conditions (12 h each). Cultures were scored for presence or absence of different fungi, including *Diaporthe* spp., based on morphology (Barnett and Hunter 1972). After 14 days, *Diaporthe* isolates were purified by transferring hyphal tips to fresh plates of PDA amended with 0.02% streptomycin sulfate.

DNA of 234 isolates collected during the 3-year survey was extracted from lyophilized mycelium scraped from the surface of 7-day-old cultures growing on PDA and resuspended in 50 µl of rehydration solution (1% Tris-EDTA buffer) using a Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI). A 5-µl aliquot of each DNA sample was electrophoresed on a 1% agarose gel to confirm quality. DNA was also quantified with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). The 234 *Diaporthe* isolates were preliminarily identified to species by amplifying and sequencing the internal transcribed spacer (ITS) regions using primers ITS5 and ITS4 (White et al. 1990). Cycle parameters included an initial denaturation at 94°C for 3 min; followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min (White et al. 1990). A 5-µl aliquot of each polymerase chain reaction (PCR) product was run electrophoretically on a 1% agarose gel to confirm amplification. All DNA samples were sequenced (McLab, San Francisco and GenScript USA Inc., Piscataway, NJ) using the respective PCR primers. Forward and reverse sequences were edited, and the contigs were aligned using Bioedit (Hall 1999). Analysis of the edited sequences was performed using the Basic Local Alignment Search Tool (BLASTN) at the GenBank nucleotide database (National Center for Biotechnology Information [NCBI]). The corresponding fungi were identified based on top BLAST results (lowest e-value, highest score, and greatest similarity). The DNA sequences from isolates that were found to have more than 95% identity with *Diaporthe* sequences in GenBank, and also to have an e-value less than e-10 in the BLASTN searches, were used for further analysis.

To establish a well-resolved phylogeny and to further clarify the phylogenetic position of *Diaporthe* spp. infecting sunflower in the

TABLE 1. Number of fields surveyed, stems collected, and *Diaporthe* isolates recovered in the Northern Great Plains in 2010, 2011, and 2012

Year	State ^a	Fields Surveyed	Total number of stems collected	Number and percentage of <i>Diaporthe</i> isolates recovered			
				<i>D. helianthi</i>	<i>D. helianthi</i> isolation (%)	<i>D. gulyae</i>	<i>D. gulyae</i> isolation (%)
2010	MN	1	6	4	66.7	0	0.0
2010	ND	6	8	7	87.5	0	0.0
2010	SD	48	76	3	3.9	69	90.8
2011	MN	14	37	27	73.0	0	0.0
2011	ND	14	23	8	34.8	0	0.0
2011	SD	23	70	59	84.3	2	2.9
2012	MN	10	26	26	100.0	0	0.0
2012	ND	5	14	14	100.0	0	0.0
2012	SD	11	15	15	100.0	0	0.0

^a MN = Minnesota, ND = North Dakota, and SD = South Dakota.

Northern Great Plains, 19 representative *Diaporthe* isolates were selected and characterized by phylogenetic analyses of two gene fragments, including translation elongation factor 1- α (EF1- α) and actin (ACT) (Table 2). An intron region of the EF1- α gene was amplified using primers EF1-728F and EF1-986R (Carbone and Kohn 1999). A region of the ACT gene spanning an intron was amplified using the primers ACT-512F and ACT-783R (Carbone and Kohn 1999). Reactions for all PCR amplifications were performed in a 25- μ l mixture containing template DNA at 20 to 30 ng/reaction, 10 μ M each primer, 10 mM each dNTP, Taq DNA Polymerase (Qiagen, Valencia, CA) at 5 units/ μ l, and 10 $\times$ Qiagen PCR Buffer containing 15 mM MgCl₂ (Qiagen). The PCR cycling protocols were denaturation at 94°C for 5 min; followed by 39 cycles of 30 s at 95°C, 50 s at 55°C (for primer pairs ACT-512F/ACT-783R) or 58°C (for primer pairs EF1-728F/EF1-986R), and 1 min at 72°C; and a final step of 10 min at 72°C (Carbone and Kohn 1999). A 5- μ l aliquot of each PCR product was electrophoresed on a 1% agarose gel to confirm amplification. All DNA samples were sequenced (McLab and GenScript USA Inc.) using the respective PCR primers. The DNA sequences that were sequenced and analyzed in this study were deposited in GenBank under accession numbers KM230818 to KM230869 and KM230901 to KM230926 (Table 2).

Molecular phylogenetics. The ITS, EF1- α , and ACT sequences of *Diaporthe* isolates were aligned using the default parameters of ClustalX (Thompson et al. 1997) and adjusted manually by visual examination using the Molecular Evolutionary Genetics Analysis (MEGA) software v5.2.2 (Tamura et al. 2011) prior to being exported as NEXUS files for subsequent analyses. Prior to the combined analyses, the concordance of the three gene datasets was evaluated with the partition-homogeneity test (Farris et al. 1994) implemented with PAUP* v4.0b10 (Sinauer Associates, Inc., Sunderland, MA) (Swofford 2002), using 1,000 random repartitions (Felsenstein 1985), with MAXTREES set to 5,000. The null hypothesis of congruence was rejected if $P < 0.001$ (Darlu and Lecointre 2002; Dettman et al. 2003). For the combined dataset, sequences were concatenated using Mesquite v2.75 (Maddison and Maddison 2011).

Phylogenies based on maximum parsimony (MP) were inferred using MEGA, and Bayesian inference (BI) using MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003), under the estimated model of evolution. For the MP analyses, heuristic searches for the most

parsimonious trees were conducted using the tree bisection-regrafting algorithm (Nei and Kumar 2000) with search level 1, in which the initial trees were obtained by random addition (10 replicates). For the MP analyses, gaps were treated as missing data and relative support for branches was estimated with 1,000 bootstrap replications (Felsenstein 1985).

For BI analyses, two simultaneous runs were conducted with Markov chain Monte Carlo (MCMC) chains with default priors and a conservative burn-in of 25% and tree sampling every 100 generations. Four parallel MCMC chains were run for 1,000,000 generations; a burn-in of 2,500 generations was found to be sufficient to achieve stationary model parameters using Tracer v1.4.1 (Rambaut and Drummond 2007). Within each run, the average standard deviation of split frequencies and the potential scale reduction factor statistics from MrBayes were used to evaluate topological and branch-length convergence, respectively.

The best-fitting evolutionary models for the Bayesian analyses were estimated using ModelTest v3.7 (Posada and Crandall 1998) by Akaike information criterion (Akaike 1974). For the ITS region, the general time-reversible model with a proportion of invariant sites (+I) and a six-category, discrete γ (+G) shape distribution (Lanave et al. 1984) was selected as the best fitting model. Sequences for the outgroup taxa *Valsa ceratosperma* (Tode) Maire (NCBI accession number AY347335) and *Leucostoma persoonii* (Nitschke) Höhn. (NCBI accession number DQ996042) were obtained from GenBank. For the combined EF1- α and ACT gene phylogenetic analyses, the best-fitting model of nucleotide substitution was the HKY+G (Yang 1994). The outgroup *Chrysospor-theella hodgesiana* Gryzenhout and M. J. (isolate CMW9995) was obtained from GenBank (NCBI accession number GQ290152 for *C. hodgesiana* EF1- α sequence and GQ290170 for ACT sequence). The 49 sequences in the combined data set (including the outgroup *C. hodgesiana*) comprised 667 bp of aligned sequence. The Bayesian posterior probabilities (PP) for each node were estimated from the resulting 50% majority-rule consensus tree and nodes with 95% or greater PP were considered significant (Wilcox et al. 2002). Each of the Bayesian MCMC analyses was run at least twice to confirm the consistency of the results. Phylogenetic trees inferred using MrBayes analyses were observed in FigTree v1.3.1 (Rambaut 2009).

Aggressiveness of *Diaporthe* spp. on sunflower. To compare aggressiveness between *Diaporthe* spp. and also among

TABLE 2. Isolates used in this study, the genes sequenced, and GenBank accessions

Isolates ^a	Year of isolation	State ^b	Species identity ^c	GenBank accession numbers		
				ITS	EF1- α	ACT
D6	2010	SD	<i>Diaporthe gulyae</i>	KM230818	KM230844	KM230901
D9	2010	SD	<i>D. gulyae</i>	KM230819	KM230845	KM230902
D12	2010	SD	<i>D. gulyae</i>	KM230820	KM230846	KM230903
D14	2010	SD	<i>D. gulyae</i>	KM230821	KM230847	KM230904
D21	2010	SD	<i>D. gulyae</i>	KM230822	KM230848	KM230905
D32	2010	SD	<i>D. gulyae</i>	KM230823	KM230849	KM230906
D40	2010	SD	<i>D. gulyae</i>	KM230824	KM230850	KM230907
R_P131	2010	MN	<i>D. helianthi</i>	KM230832	KM230858	KM230915
R_P129	2010	ND	<i>D. helianthi</i>	KM230833	KM230859	KM230916
R_P132	2011	MN	<i>D. helianthi</i>	KM230834	KM230860	KM230917
R_P126	2011	MN	<i>D. helianthi</i>	KM230835	KM230861	KM230918
R_P121	2011	MN	<i>D. helianthi</i>	KM230836	KM230862	KM230919
R_P105	2011	MN	<i>D. helianthi</i>	KM230837	KM230863	KM230920
R_P140	2011	MN	<i>D. helianthi</i>	KM230838	KM230864	KM230921
R_P137	2011	MN	<i>D. helianthi</i>	KM230839	KM230865	KM230922
R_P134	2012	MN	<i>D. helianthi</i>	KM230840	KM230866	KM230923
R_P118	2012	MN	<i>D. helianthi</i>	KM230841	KM230867	KM230924
R_P139	2012	MN	<i>D. helianthi</i>	KM230842	KM230868	KM230925
R_P107	2012	MN	<i>D. helianthi</i>	KM230843	KM230869	KM230926

^a Isolates are a subset of 234 *Diaporthe* isolates and were chosen as representatives for species-level identification.

^b SD = South Dakota, MN = Minnesota, and ND = North Dakota.

^c Species identity was established based on phylogenetic analysis of the internal transcribed spacer region (ITS), elongation factor subunit 1- α (EF1- α), and actin (ACT).

isolates within the two species, 10 isolates each of *D. helianthi* and *D. gulyae* were selected as representative of each species. These isolates included 10 of *D. gulyae* from South Dakota, 8 of *D. helianthi* from Minnesota, 1 of *D. helianthi* from North Dakota, and 1 of *D. helianthi* from South Dakota. Isolates of *Diaporthe* spp. were cultured on PDA amended with 0.02% streptomycin sulfate to avoid bacterial growth. Plates were incubated at room temperature for 10 days under 12-h cycles of light and darkness. Mycelial plugs (4 mm in diameter) were taken from the margin of the growing colony.

The United States Department of Agriculture (USDA) confection inbred line 'HA 288' is considered universally susceptible to *D. helianthi*, and was selected as the host genotype for the study. Two seeds were planted in moist planting mix (Sunshine Mix number 1; Sun Grow Horticulture Products, Bellevue, WA) in 7.5-liter circular plastic pots. The pots were placed in a greenhouse with a temperature of 22 to 25°C and a 16-h cycle of light and darkness, and were watered on alternate days. Based on the results of an experiment to determine the most effective inoculation method of *Diaporthe* spp. on sunflower (data not published), the stem-wound method was selected to compare aggressiveness. When the plants were between the V4 and V6 growth stages (four to six true leaves), the stems were wounded with a micropipette tip (100 µl) on the second internode and a *Diaporthe*-infested mycelial plug was placed on the wound. A sterile PDA plug was placed on the wound of the nontreated control. After inoculation, the plants were returned to the same greenhouse conditions. Disease severity was evaluated at 3, 10, and 14 days after inoculation using a 0-to-5 scale modified from Thompson et al. (2011), where 0 = no discoloration; 1 = low level discoloration at site of inoculation; 2 = slight discoloration or lesion 1 to 2 mm in length; 3 = necrotic lesions 2 to 5 mm in length, some colored stem streaking, leaf wilting, and twisting; 4 = lesions 5 to 10 mm in length, significant necrosis and dark-colored stem streaking, leaf and plant wilting, twisting, stunting, and some lodging; and 5 = lesions exceeding 10 mm in length, severe leaf necrosis, lodging, or plant death.

The trial was conducted in a completely randomized design with 12 plants evaluated per treatment, and the experiment was repeated twice. The experimental unit was the single plant in each pot and each treatment was replicated 12 times (12 plants). Analysis of data based on observed ranks was performed in SAS. The ordinal data from the disease rating scale did not have a normal distribution; therefore, they were analyzed using the nonparametric procedure of Brunner et al. (2002) as described by Shah and Madden (2004). Preliminary data analysis showed that the results of both experimental runs were very similar; therefore, the data from both runs were combined for the final analysis. A repeated-measures analysis was used to identify significant effects of time on the disease produced by the two *Diaporthe* spp. and isolates within species. The overall and interaction effect of *Diaporthe* spp., isolates within species, and time on disease severity was determined by the ATS of ranked data. The PROC RANK procedure in SAS was used to obtain the mean treatment rank (R) followed by PROC MIXED to generate relative effects (RE). According to Shah and Madden (2004) and Akritas (1991), the mean treatment rank (R) for the *i*th treatment is defined by the equation $R = \frac{1}{n} (\sum (k-1) n_i)$ R_i , where R_i is the rank among all *N* observations and n_i indicates independent experimental units or replications ($k = 1, \dots, n_i$) per treatment. RE, defined by means of probabilities derived from the marginal distribution functions of disease severity, are generated by the equation $RE = (R - 0.5)/N$, where *R* is the mean treatment rank and *N* is the total number of observations in the data analysis. Confidence intervals were calculated using LD_CI macro in SAS at $P \leq 0.05$ (Brunner et al. 2002).

In addition to comparing aggressiveness of the two *Diaporthe* spp. and isolates within the two species, the experiment was also used to complete Koch's postulates of *D. gulyae*, which was not

previously reported as a pathogen of sunflower causing Phomopsis stem canker in the United States.

RESULTS

Survey collection and identification of *Diaporthe* isolates. During the 3-year survey, two species of *Diaporthe* were isolated from infected sunflower stems collected from the Northern Great Plains (Table 1). The frequency of *D. helianthi* and *D. gulyae* varied among the 3 years and among the three states. In 2010, *D. helianthi* was isolated at frequencies of 3.9% from samples collected in South Dakota, 66.7% from samples collected in Minnesota, and 87.5% from samples collected in North Dakota. However, *D. gulyae* was isolated at a frequency of 90.8% from samples collected in South Dakota (Table 1). In 2011, *D. helianthi* was isolated at a frequency of 73.0% from samples collected in Minnesota, 34.8% North Dakota, and 84.3% in South Dakota, whereas *D. gulyae* was isolated at frequency of 2.9% from samples collected in South Dakota (Table 1). In 2012, only *D. helianthi* was isolated (Table 1).

An approximately 600-bp region of the ITS gene region was amplified from 234 isolates originating from the United States, and was used to query the GenBank database directly. A BLASTN search of GenBank was performed for the ITS sequences of 163 U.S. isolates identified as *D. helianthi* based on comparison with the type isolate from sunflower (*D. helianthi* strain CBS 592.81, accession number AY705842) as designated by Muntanola-Cvetković et al. (1985) (Table 2). These isolates differed at a single nucleotide site from the type isolate for the ITS gene. A BLASTN search of GenBank was performed for the ITS sequences of the *D. gulyae* U.S. isolates (72 isolates, 30.4%), which showed that the best match was *Phomopsis* sp. AJY-2011a strain T12505G (*D. gulyae*, accession number JF431299) from *H. annuus* in Australia, with identities = 540/540 (100%) and gaps = 0/540 (0%) (Table 2).

Molecular phylogenetics. For sequences of the 19 *Diaporthe* isolates selected as representative of the U.S. population, Bayesian and MP analyses produced similar topologies in analyses of each locus alone (ITS) and combined (EF1- α and ACT). In the ITS tree, of the 19 *Diaporthe* isolates, 12 isolates clustered with the ex-type culture of *D. helianthi* (CBS 592.81, accession number AY705842) and 7 isolates with the ex-type culture of *Phomopsis* sp. AJY-2011a strain T12505G (*D. gulyae*, MycoBank MB561569, accession number JF431299) in phylogenetic analyses of the ITS data set (data not shown). The *D. helianthi* isolates from the Northern Great Plains clustered (PP = 90%) with the *D. helianthi* isolates from *H. annuus* in Croatia, France, Yugoslavia, and the United States in the ITS tree. In contrast, *D. gulyae* collections from the Northern Great Plains were placed in the group containing *D. gulyae* isolates from *H. annuus* in Australia as well as the type sequences of *D. ambigua*, *D. angelicae*, *D. stewartii*, *D. dauci*, *D. longicolla*, and *D. phaseolorum*. The *D. gulyae* clade appears as a moderately well-supported branch, with 70% PP in the ITS tree, compared with 100% PP for the well-established *D. helianthi* clade (data not shown). Because the ITS tree was not well resolved for Bayesian and MP analyses and it gave no additional information not already addressed by the BLAST search, the ITS tree was not included in this article.

For further characterization of the *Diaporthe* isolates using combined genes, EF1- α and ACT genes were combined based on the results of the partition-homogeneity test ($P = 0.433$) indicating that the trees reflect the same underlying phylogeny. The MP analysis for the combined tree resulted in one most parsimonious tree (length = 269, consistency index [CI] = 0.788, retention index [RI] = 0.965, and rescaled consistency index [RCI] = 0.789) for all sites and parsimony-informative sites (Fig. 1). Overall, there was an increase in bootstrap support in the resulting phylogenetic tree when the combined dataset was used (Fig. 1). Whereas *D. gulyae* formed

a monophyletic group with the type strains DG54025 and DG54026 (PP = 100%), *D. helianthi* isolates clustered together with the type strain CBS 592.81 and was supported by an overall of PP of 100% (Fig. 1).

Aggressiveness of *Diaporthe* spp. The disease symptoms caused by the two *Diaporthe* spp. were evaluated at 3, 10, and 14 days after inoculation using RE and their 95% CI. Test statistics indicated no significant effect of experiment or interaction effects between experiment and *Diaporthe* spp. in the overall development of Phomopsis stem canker on sunflower. The repeated-measures analysis identified a significant interaction between *Diaporthe* spp. and time at $P = 0.0009$. The interaction was because *D. gulyae* and *D. helianthi* differed in their RE at 3 days after inoculation (Table 3). However, there were no significant differences in RE between the two *Diaporthe* spp. at 10 and 14 days after inoculation (Fig. 2;

Table 3). Based on visual symptoms, *D. gulyae* caused brown lesions extending upward from the inoculation site, wilting of stem and leaves at the node closest to the site of inoculation 3 days after inoculation, and plant death. In contrast, *D. helianthi* caused tan to brown elongated lesions 10 days after inoculation and no plant death was observed. No symptoms were observed in the control plants.

The repeated-measures analysis also identified a significant interaction among *Diaporthe* isolates (within the two species) and time at $P < 0.0001$. Among *D. gulyae* isolates, significant differences in their RE was more evident at 3 days after inoculation compared with 10 and 14 days after inoculation (Supplementary Table S1). In contrast, among *D. helianthi* isolates, significant differences in their RE were evident at 3 and 10 days after inoculation compared with 14 days after inoculation. For example,

Fig. 1. Phylogenetic tree resulting from the alignment of 667 characters of the combined EF1- α and ACT gene region using the Bayesian inference and maximum parsimony analyses of the *Diaporthe* isolates from sunflower. Branch labels represent bootstrap support values from maximum parsimony analyses (≥ 70) over Bayesian posterior probability values (≥ 0.70). *Diaporthe* isolates recovered from sunflower fields in the Northern Great Plains have labels ‘DH’ for *Diaporthe helianthi* (CBS 592.81 = type strain) and ‘DG’ for *Diaporthe gulyae* (DG54025 and DG54026 = type strains). *Chrysosportheella hodgesiana* (isolate CMW9995) was used as the outgroup (National Center for Biotechnology Information accession number GQ290152 for EF1- α sequence and GQ290170 for ACT sequence).

TABLE 3. Median, mean rank and relative treatment effects for Phomopsis stem canker severity rating caused by two *Diaporthe* spp. on sunflower at 3, 10, and 14 days after inoculation

Species	Days after inoculation								
	Median disease rating ^a			Mean rank			Estimated relative effect ^b		
	3	10	14	3	10	14	3	10	14
<i>Diaporthe gulyae</i>	2.5	3.0	4.0	257.9	378.5	495.0	0.36 (0.32,0.39)	0.53 (0.49,0.56)	0.69 (0.66,0.71)
<i>D. helianthi</i>	2.0	3.0	3.0	194.2	370.2	370.2	0.27 (0.24,0.30)	0.51 (0.47,0.56)	0.65 (0.62,0.67)

^a Disease severity ratings were evaluated at 3, 10, and 14 days after inoculation using a 0-to-5 scale modified from Thompson et al. (2011).
^b Relative treatment effects were calculated using the nonparametric method for ordinal data described by Shah and Madden (2004).

the RE of DH10 was lower than that of DH7, DH8, and DH9 at 3 and 10 days after inoculation; however, the RE of the four *D. helianthi* isolates were not significantly different in their RE at 14 days after inoculation.

To complete Koch's postulates, *D. gulyae* was reisolated from the inoculated HA 288 stems 14 days after inoculation and the identity of the pathogen was confirmed via sequencing of the ITS regions using primers ITS5 and ITS4 (White et al. 1990). The pathogen was not isolated from the control plants.

DISCUSSION

The results of this study demonstrated that two *Diaporthe* spp. (*D. helianthi* and *D. gulyae*) are responsible for Phomopsis stem canker on sunflower in the United States. The identification of *Diaporthe* spp. causing Phomopsis stem canker was based on DNA sequence analyses of the ITS, EF-1 α , and ACT genes because morphological characteristics are unreliable (van Rensburg et al. 2006). Phylogenetic analysis of ITS sequences of *D. helianthi* isolates from the United States represent a tight monophyletic clade and revealed that they were closely related to all isolates from France and the former Yugoslavia, where yield losses up to 40 and 50%, respectively, due to Phomopsis stem canker have been reported (Carré 1993; Pentericci 1988; Rekab et al. 2004). In contrast, *D. gulyae* isolates from the United States appeared as a moderately well-supported clade based on ITS sequences (data not shown). The consensus BI tree and MP tree topology inferred from the combined dataset identified two independent clusters for *D. helianthi* and *D. gulyae* supported by higher measures of clade support of 100 and 88% PP, respectively (Fig. 1). When the aggressiveness of the two *Diaporthe* spp. was compared in greenhouse conditions, *D. gulyae* produced symptoms consistent with Phomopsis stem canker more quickly than *D. helianthi*; however, both were equally damaging pathogens over time. To the best of our knowledge, this is the first report of *D. gulyae* causing Phomopsis stem canker on sunflower in the United States, and its identification may affect the development and deployment of management tools for the disease.

The existence of two *Diaporthe* spp. on sunflower in the United States could be explained by several hypotheses. Because *D. helianthi* and *D. gulyae* can be transmitted through seed (Herr et al. 1983; Thompson et al. 2011), one hypothesis would suggest a recent introduction of new species on seed. *D. helianthi* was confirmed by morphology in Ohio in 1980 (Herr et al. 1983), followed by Texas in 1982 (Yang et al. 1984) and Minnesota and North Dakota in 1984 (Hajdu et al. 1984). Although we did not have

any *Diaporthe* isolates from sunflower prior to 2010 for use in our molecular study, we were able to perform Bayesian analyses of the ITS sequences deposited by Miric (2002) in the GenBank nucleotide database of the two isolates (from North Dakota) collected in 1984 and 1988 (data not presented). The isolates were identified as *D. helianthi* based on comparison with the type isolate strain CBS 592.81 (accession number AY705842). Although this eliminates the hypothesis of a recent introduction of *D. helianthi* into the United States, we did not confirm the same for *D. gulyae*.

Another hypothesis is that both *Diaporthe* spp. were endemic in the United States. Herr et al. (1983) reported that *Diaporthe* isolates identified causing Phomopsis stem canker in Ohio produced pycnidia with predominantly α -conidia and some with predominantly β -conidia or those with both the conidia types. They concluded that the *Diaporthe* isolates that produced predominantly β -conidia were similar to *D. helianthi*, as described in Yugoslavia. However, they were not able to resolve inconsistencies in the description of the *Diaporthe* isolates found in Ohio in comparison with those from Yugoslavia (Mihaljčević et al. 1985), particularly for those that produced predominantly α -conidia. In our study, *D. helianthi* isolates produced white, floccose, and dense mycelia, dark-brown colored, on PDA; size of β -conidia (20 to 35 by 0.5 to 2.7 μ m) was consistent with Gulya et al. (1997). In contrast, *D. gulyae* appeared flat in the center of the PDA plate and irregularly fluffy near the margin, with few aerial hyphae, and the size of α -conidia (6.5 to 8.5 by 2.5 to 3.5 μ m) was fairly consistent with Thompson et al. (2011). We did not observe β -conidia in *D. gulyae*. Additionally, *D. gulyae* caused leaf wilting, necrosis, and lodging in our aggressiveness study; these matched the symptoms described by Herr et al. (1983) but not those described by Mihaljčević et al. (1985) for *D. helianthi*. Comparison of the biological differences among *Diaporthe* isolates described by the previous researchers and in our study suggests that the two *Diaporthe* spp. were already present in the United States at low levels but the ability to distinguish them prior to molecular techniques did not exist.

The present study does suggest that *D. helianthi* was more prevalent than *D. gulyae* in the Northern Great Plains, and that they were not distributed in equal amounts across years or states. State and year variation between occurrences of *Diaporthe* spp. is likely a result of differences in sampling strategies as opposed to actual distribution of the two species. For instance, in 2010, sampling of stalks was based on the appearance of any stem disease and associated symptoms such as wilting, lodging, and plant death. In 2011 and 2012, sunflower stalks were sampled from fields heavily infected by Phomopsis stem canker. In these fields, *D. helianthi* was most frequently identified, which may suggest that *D. helianthi* may

Fig. 2. Phomopsis stem canker symptoms produced by *Diaporthe gulyae* (left) and *Diaporthe helianthi* (right) on HA 288 sunflower under greenhouse conditions using stem-wound method at 14 days after inoculation. The control (center) produced no discoloration when inoculated with noninfested potato dextrose agar plug and was not included in the statistical analyses.

be more of an economic concern for sunflower production in the Northern Great Plains than *D. gulyae*. However, year-to-year variation between species occurrence has been documented previously in pathogens such as *Fusarium* spp., and this was attributed to inoculum source and overwintering structures (Backhouse and Burgess 2002). For *Diaporthe* spp., although the perithecium is known to survive in debris for 5 years (Herr et al. 1983), there are no studies evaluating the influence of environmental conditions and survival of perithecia on the ability of the fungus to develop and persist as a pathogen. Although it is likely that environmental conditions or survival of perithecia may have an influence on the distribution of the two *Diaporthe* spp. in the Northern Great Plains, this was beyond the scope of our study.

This study is the first to compare the relative overall aggressiveness of the two *Diaporthe* spp., and results suggest that, although both are aggressive pathogens, *D. gulyae* was capable of causing necrosis, lodging, and plant death at a faster rate than *D. helianthi*. Similarly, isolates of *D. gulyae* and *D. helianthi* varied significantly in their aggressiveness within the two *Diaporthe* spp. *D. helianthi* produces toxins, including phomozin, which can result in premature leaf senescence, plant wilting, and lodging (Masirevic and Gulya 1992). Although there are no studies characterizing toxins produced by *D. gulyae*, the involvement of a toxin in the disease may explain these differences in terms of the varying aggressiveness between the two species and their individual isolates at specific times; for instance, 3 days after inoculation for *D. gulyae* and 10 days after inoculation for *D. helianthi*. Given that both *Diaporthe* spp. are present in the United States, knowledge of the relative aggressiveness of the two *Diaporthe* spp. may have important implications for the success of future breeding efforts and resistance deployment.

Prior to the reemergence of Phomopsis stem canker in 2010, breeding for resistance to the disease in the United States was likely not possible in natural field environments. Since then, public and private breeding programs have experienced natural Phomopsis stem canker epidemics and are actively screening germplasm for resistance. Sunflower lines resistant to *D. helianthi* were recently identified under natural disease pressure in field conditions (Talukder et al. 2014) but it is unclear whether the resistance in those lines is also conferred to *D. gulyae*. Breeders in the private sector are actively culling very susceptible germplasm when nurseries are affected by Phomopsis stem canker but rarely attempt to have the *Diaporthe* spp. inciting the disease identified. Given that *D. gulyae* and *D. helianthi* are both aggressive and present, it is very important to incorporate resistance that is effective against both pathogens. Although screening and culling efforts due to Phomopsis stem canker is an important first step to managing this disease, active incorporation of resistance effective against both pathogens is critical for future disease management.

This study was initiated in response to an increase in incidence, severity, and reports of yield losses to Phomopsis stem canker in the Northern Great Plains. Changes in the *Diaporthe* populations in the United States may have contributed to this recent emergence of the disease but our results suggest that it is more likely that an increase in disease is a result of other factors, and that the simultaneous identification of *D. gulyae* is circumstantial. The increase in Phomopsis stem canker could be partially explained by an increased production of sunflower on no-till and minimum-tillage field ground, which could contribute to an increase in inoculum (Kandel 2012), or breeding in the absence of disease, which can inadvertently generate very susceptible hybrids. Additionally, some of the reported increases in Phomopsis stem canker incidence may be due to an increased ability among agronomists to accurately distinguish the disease from other common stem diseases, such as Phoma black stem (caused by *Phoma macdonaldii* Boerema) (Kandel 2012).

Although it is unclear which factors have facilitated the reemergence of Phomopsis stem canker as a major yield-limiting

disease in the United States, the potential impact on sunflower production cannot be overstated. The incidence of Phomopsis stem canker across the U.S. Northern Great Plains remains approximately 10-fold higher than a decade ago. Yield losses due to Phomopsis stem canker have reached 50% in noninoculated and nonirrigated research trials (Gilley et al. 2015; Mathew et al. 2015) and are thought to have been equally high in severely infected commercial fields. The presence of Phomopsis stem canker was recently determined to be a one of the most significant causes of genotype–environment variation in an ongoing study to understand sunflower crop testing environments; a notable find given the wide geography of the study and numerous yield-limiting factors affecting sunflower (B. Hulke, *personal communication*). International movement of sunflower seed could be influenced by the emergence of this disease, because *D. helianthi* is a quarantined pathogen in most countries but *D. gulyae* is not. The abrupt reemergence of Phomopsis stem canker is a new yield-limiting threat to sunflower growers in the United States, and an equally fast response by pathologists and breeders to develop and deploy management tools is needed to manage this disease.

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