Research

Comparison of Greenhouse-Based Inoculation Methods to Study Aggressiveness of *Diaporthe helianthi* Isolates Causing Phomopsis Stem Canker of Sunflower (*Helianthus annuus*)

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Abstract

Phomopsis stem canker is an economically important disease of sunflower (*Helianthus annuus*), and *Diaporthe helianthi* is one of the primary causal agents of the disease in the United States. The objective of this study was to evaluate inoculation methods of *D. helianthi* isolates on sunflower in the greenhouse. Four isolates of *D. helianthi* were selected to test the effectiveness of four inoculation methods using mycelial plugs as the inoculum, including stem wound, wound inoculation, petiole wound, and straw test.

Infection was established by the *D. helianthi* isolates at 14 days after inoculation for all inoculation methods used. However, recovery of the pathogen from the inoculated plants differed significantly ($P < 0.0001$) among inoculation methods. Given higher mean recovery of *D. helianthi* isolates from the inoculated plants and the size of the lesions caused by the pathogen, the stem-wound inoculation method was found to be the most user friendly of the four inoculation methods.

Phomopsis stem canker of sunflower (*Helianthus annuus* L.) in the United States is caused by three species of *Diaporthe*, namely, *Diaporthe helianthi* Muntañola-Cvetkovic, Mihaljcevic and Petrov (Mathew et al. 2015; Muntañola-Cvetković et al. 1985; Yang et al. 1984), *Diaporthe gulyae* Shivas, Thompson and Young (Mathew et al. 2015; Thompson et al. 2011), and *Diaporthe stewartii* Harrison (Olson et al. 2017). Among the three species, *D. helianthi* is the predominant pathogen in the United States (Mathew et al. 2015). The disease begins as a foliar infection, quickly spreads through the petiole, and produces stem lesions that are typically dark brown, greater than 15 cm in length, and often girdle the stem (Markell et al. 2015a, b) (Fig. 1). The pith beneath the stem lesion becomes hollow, and the loss of stem integrity frequently results in lodging (Fig. 2). Historically, the disease has been one of the primary yield-limiting factors for sunflower production in Europe, where yield losses of up to 50% have occurred (Harveson et al. 2016; Masirevic and Gulya 1992). In the United States, Phomopsis stem canker has had a more limited impact on sunflower production. However, the prevalence of Phomopsis stem canker increased from 1.5% in 2001 to 61.0% in 2015 in areas of the U.S. Northern Great Plains (Gulya and Kandel 2016). Up to 50% yield reductions owing to Phomopsis stem canker were reported from commercial fields and seed nurseries in northern Minnesota and North Dakota between 2010 and 2012 (Mathew et al. 2015). In response to the disease epidemic, the U.S. National Sunflower

Association (NSA) made management of Phomopsis stem canker one of their top research priorities in the 2010s.

Phomopsis stem canker can be partially managed with genetic resistance, and effective methods to screen germplasm for resistance are critical for the development of resistant hybrids (Berglund 2007; Markell et al. 2015b). Although ascospores produced by species of *Diaporthe* are widely regarded as the primary source of inoculum for Phomopsis stem canker of sunflower, the in vitro production of ascospores is difficult. This has limited the ability of plant pathologists and plant breeders to screen sunflower germplasm under conditions that best mimic the disease cycle (Viguié et al. 1999). Several artificial inoculation methods have been developed to study *Diaporthe* aggressiveness and screen sunflower germplasm for resistance (Encheva and Kiryakov 2002; Thompson et al. 2011; Tourvieille et al. 1988). All of these methods used mycelial plugs as the source of inoculum. For example, Encheva and Kiryakov (2002) investigated the use of the straw method for testing sunflower response to *D. helianthi*. Briefly, plants were inoculated at the bud stage (Schneiter et al. 1998) by cutting the leaf petiole with a scalpel and inserting a plastic straw containing an agar plug of a 7-day-old *D. helianthi* culture. A significant correlation was observed between disease severity from the straw test and disease severity from natural infection by ascospores (Encheva and Kiryakov 2002). Thompson et al. (2011) used two inoculation methods (wound inoculation and mycelium contact) to quantify the severity of Phomopsis stem canker of sunflower caused by *D. gulyae*, by *D. kongii* Shivas, Thompson and Young, and by *D. kochmanii* Shivas, Thompson and Young (Thompson et al. 2011). For the wound-inoculation method, plants were inoculated at the 6 to 8 true leaves (V6 to V8) (Schneiter et al. 1998) growth stage by making a 5 to 10 mm slit on a node with a scalpel, placing an agar plug of 7-day-old *Diaporthe* culture into the slit, and wrapping the plug with Parafilm. Their mycelial contact

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method involved placing an inoculated agar plug in contact with the stem at a node and wrapping the plug with Parafilm (Thompson et al. 2011). Thompson et al. (2011) concluded that the wound and mycelium contact methods produced similar results for individual isolates of *D. gulyae*, *D. kongii*, and *D. kochmanii* at 14 days after inoculation, although sunflower plants inoculated by wounding showed symptoms of Phomopsis stem canker 1 to 7 days earlier than those inoculated by the mycelium contact (nonwounding) method.

Although several inoculation methods to study aggressiveness of *Diaporthe* species have been used, qualitative comparisons of these methods is lacking. The objective of this study was to evaluate the effectiveness of four methods to screen aggressiveness of *D. helianthi* isolates, the predominant pathogenic species in the United States, for their aggressiveness on sunflower in the greenhouse.

Explanation of the Four Methods and the Experimental Design Used to Evaluate Them

To determine the most effective greenhouse-based inoculation technique, four inoculation methods that involved wounding of the stem or petiole were evaluated, namely, stem wound, petiole wound, wound inoculation, and straw test (Encheva and Kiryakov 2002; Thompson et al. 2011). A nonwounding method was not included in our study because it was observed by Thompson et al. (2011) that Phomopsis stem canker symptoms developed more slowly with this method.

For this study, a factorial experiment arranged in a completely randomized design with combinations of four inoculation methods and four *D. helianthi* isolates was established. Twelve plants (V6 to V8 growth stage; Schneiter et al. 1998) were evaluated in each combination of inoculation method and isolate. The experiment was repeated once. The experiment was conducted using the USDA sunflower confection inbred line 'HA 288', which is considered highly susceptible to *D. helianthi* (Mathew et al. 2015). Two seeds of HA 288 were planted in moist planting mix (Sunshine Mix number 1, Sun Grow Horticulture Products, Bellevue, WA) in 7.5-liter circular plastic pots. A total of 96 pots were planted for each experiment, with plants in six pots (12 plants total) evaluated for each method (4) × isolate (4) combination. The pots were placed on greenhouse benches under a 16-h photoperiod at $25 \pm 2^\circ\text{C}$ and watered on alternate days.

Four isolates of *D. helianthi* were selected, based on results of a preliminary aggressiveness greenhouse study (F. Mathew and S. Markell, *unpublished*) and on geographic origin. These included two isolates from Minnesota in 2011 (DH1 and DH4), one from North Dakota in 2010 (DH2), and one from South Dakota in 2011 (DH3) (Table 1). The four selected *D. helianthi* isolates were originally recovered from symptomatic sunflower plants sampled during a survey by Mathew et al. (2015) and identified as *D. helianthi* based on morphological characteristics as well as by sequencing the

FIGURE 1
Phomopsis stem canker lesions on a sunflower stem.

FIGURE 2
Lodged sunflower caused by Phomopsis stem canker lesion.

TABLE 1
Median, mean rank, and relative treatment effects for *Phomopsis* stem canker severity on sunflower (cultivar HA 288) caused by *Diaporthe helianthi* isolates using four inoculation methods

Inoculation method ^a	Inoculum ^b	Median disease rating ^c	Mean rank	Estimated relative effect (RE) ^d
Petiole wound	DH1	2.0	85.0	0.35 (0.34, 0.37)
	DH2	2.5	139.0	0.58 (0.45, 0.69)*
	DH3	3.0	193.0	0.80 (0.79, 0.81)*
	DH4	3.0	193.0	0.80 (0.79, 0.81)*
	Noninfested control	2.0	85.0	0.35 (0.34, 0.37)
Stem wound	DH1	3.0	193.0	0.80 (0.79, 0.81)*
	DH2	2.0	85.0	0.35 (0.34, 0.37)
	DH3	2.0	85.0	0.35 (0.34, 0.37)
	DH4	2.0	85.0	0.35 (0.34, 0.37)
	Noninfested control	1.0	36.7	0.15 (0.09, 0.25)*
Straw test	DH1	3.0	193.0	0.80 (0.79, 0.81)*
	DH2	3.0	193.0	0.80 (0.79, 0.81)*
	DH3	2.5	139.0	0.58 (0.45, 0.69)
	DH4	3.0	184.0	0.76 (0.69, 0.83)
	Noninfested control	1.0	36.7	0.15 (0.09, 0.25)*
Wound inoculation	DH1	3.0	193.0	0.80 (0.79, 0.81)*
	DH2	2.0	85.0	0.35 (0.34, 0.37)
	DH3	2.0	85.0	0.35 (0.34, 0.37)
	DH4	2.0	85.0	0.35 (0.34, 0.37)
	Noninfested control	1.0	36.6	0.15 (0.09, 0.25)*

^a In each method used, agar plugs were affixed to sunflowers with wounds, including a linear slit made by a scalpel on the second internode (wound inoculation) and on a petiole (straw test) and by a puncture wound made by a micropipette tip on the second internode (stem wound) and a petiole (petiole wound) according to Encheva and Kiryakov (2002), Thompson et al. (2011), and Tourvieille et al. (1988).

^b Inoculum was potato dextrose agar plugs with *D. helianthi* isolates DH1 (originating from Minnesota, 2011), DH2 (North Dakota, 2010), DH3 (South Dakota, 2011), DH4 (Minnesota, 2011), and a noninfested agar plug used as a control.

^c Stem lesion size was evaluated at 14 days after inoculation using a 0 to 5 scale modified from Thompson et al. (2011): 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, and 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.

^d RE and confidence interval (95%, in parentheses) were calculated using the nonparametric method for ordinal data described by Shah and Madden (2004). Asterisk (*) indicates that the RE is significantly different among treatments ($P \leq 0.05$).

internal transcribed spacer (ITS) gene region using primers ITS4 and ITS5 (White et al. 1990) and translation elongation factor 1- α (EF1- α) gene region using primers EF1-728F and EF1-986R (Carbone and Kohn 1999). To generate inoculum, isolates were grown on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) plates that were incubated at 22°C for 10 days under 12 h of alternating light and dark conditions. Mycelial plugs (4 mm in diameter) taken from the margin of the growing colonies were used as inoculum. A noninoculated control was included for each of the inoculation methods, wherein the sunflower plants were inoculated with sterile plugs.

For the wound-inoculation method, inoculation points were made on each stem by making a vertical slit on the second internode of ~5 mm in length using a sterile scalpel (Thompson et al. 2011). A mycelial plug of the four *D. helianthi* isolates was placed into the slit made in the stem and wrapped with Parafilm (Pechiney Plastic Packaging, Chicago, IL) to avoid a rapid dehydration. Control plants were similarly wounded, inoculated with noninfested PDA plugs, and wrapped in Parafilm.

For the stem- and petiole-wound experiments, a puncture wound was made with micropipette tips (100 μ l) on the second internode and on the petiole, respectively. A mycelial plug of one of the four *D. helianthi* isolates was placed over the wounded stalk or petiole. Control plants were wounded with micropipette tips on the stem and petiole, the same as for the treated plants, and inoculated with noninfested PDA plugs. All plugs were attached to the wounds with Parafilm to avoid rapid dehydration.

For the straw test (Encheva and Kiryakov 2002), sunflowers plants were inoculated by cutting the leaf petiole located at the middle of the plant (~30 mm from the stem) with a scalpel. A micropipette tip containing an agar plug of 10-day-old culture of one of the four *D. helianthi* isolates was placed over the cut sunflower petiole. Micropipette tips containing plugs of noninfested PDA served as the control. All plugs were held in place with Parafilm to avoid rapid dehydration.

Stem lesion size was evaluated at 14 days after inoculation using a 0 to 5 scale: 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, and 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 (Mathew et al. 2015; Thompson et al. 2011).

Test statistics were calculated in SAS (version 9.3, SAS Institute, Cary, NC) using the nonparametric methods of Brunner et al. (2002) as described by Shah and Madden (2004), because the disease severity scale used was an ordinal scale of measurement (rating scale of 0 to 5). The overall and interaction effect of inoculation methods and *D. helianthi* isolates was determined by the analysis of variance type statistic of ranked data, which has an approximate *F* distribution under the null hypothesis (Singer et al. 2004). The PROC RANK procedure in SAS was used to obtain median and ranks (which is defined as the number of observations

that are less than or equal to x , where x is an observation in a given set of observations). The PROC RANK procedure was followed by PROC MIXED in SAS to generate relative treatment effects (REs; also known as nonparametric treatment effects) and significance levels. REs are defined by means of probabilities derived from the marginal distribution functions of the response variable and were generated by the following equation: $RE = (R - 0.5)/N$, where R is the mean rank of the treatments and N is the total number of observations in the data analysis (Singer et al. 2004). In our study, the null hypothesis (H_0) was that the REs of all treatments were the same, and the alternative hypothesis (H_A) was that at least one of the REs was different from the other treatments. To compare treatments using RE, confidence intervals (CIs) were calculated using the LD_CI macro (Brunner et al. 2002) in SAS at $P \leq 0.05$.

Interactions Between Methods and Isolates

A significant interaction was observed between inoculation methods and *D. helianthi* isolates ($P < 0.0001$), indicating that the inoculation method influenced the disease caused by the *D. helianthi* isolates. Therefore, variation in REs among the *D. helianthi* isolates within each inoculation method was evaluated. The interaction of the main effects, specifically inoculation methods and isolates, was owing to the differences in aggressiveness among *D. helianthi* isolates resulting from the different inoculation methods. For instance, disease severity (expressed in terms of RE) caused by isolates DH3 and DH4 was significantly higher ($P \leq 0.05$) than the other isolates using the petiole-wound method (Table 1). In contrast, disease severity caused by isolates DH3 and DH4 was significantly lower ($P \leq 0.05$) than the other isolates when using the straw test (Table 1). Using the stem-wound and wound-inoculation methods, disease severity (significant at $P \leq 0.05$) caused by isolate DH1 was higher than the other isolates (Table 1).

Effectiveness and Differences Among the Four Methods

All four inoculation methods resulted in infection on the stems or petioles. At 14 days after inoculation, visible brown to reddish-brown lesions on stalks were evaluated. Symptoms produced on methods that directly wounded the stem (stem-wound and wound-inoculation) produced large and rapidly expanding lesions. Symptoms on plants inoculated on the petiole (petiole-wound and straw methods) resulted in lesions that reached the main stem from the petiole and sometimes caused wilting when the lesions expanded transversely toward the main stem. All inoculated plants, with the exception of plants inoculated using the petiole-wound method with isolate DH1, had disease severity (expressed in terms of RE) significantly different from the control (Table 1), indicating that all inoculation methods facilitated disease development.

An additional assessment of inoculation methods was made by determining the recovery of *D. helianthi* from the inoculated plants. At 14 days after inoculation, each of the inoculated plants was chopped, and ~10 mm long pieces were cut from infected tissue after the samples were washed with tap water for 2 min. The pieces were sequentially surface sterilized in sodium hypochlorite (0.05%) 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 PDA plates amended with 0.02% streptomycin sulfate. Plates were incubated at 22°C for 10 days under 12 h of alternating light and dark conditions. Cultures were hyphal-tip purified, and pathogen identity was confirmed via sequencing of the ITS gene region using ITS4 and ITS5 primers (White et al. 1990) and by comparing ITS sequences of the recovered isolates with that of *D. helianthi* isolate CBS 592.81 (GenBank accession no. NR_103698.1).

For each method, recovery of *D. helianthi* (%) was averaged across four isolates and compared in order to make a qualitative comparison of the four inoculation methods. Mean recovery of *D. helianthi* differed significantly (LSD = 18.2; $P < 0.0001$) among the inoculation methods. The highest mean recovery of *D. helianthi* was obtained from plants inoculated using the stem-wound inoculation method (73.3%) and wound-inoculation method (68.3%). The straw test and petiole-wound method had lower recovery of *D. helianthi* (33.3 and 31.7%, respectively), and these two methods were not significantly different. The pathogen was not isolated from the control plants.

Although typical symptoms of Phomopsis stem canker (light brown lesion on stem and petiole) were observed using all inoculation techniques, the stem-wound method seemed to be the most efficient based on lesion size and recovery of *D. helianthi* from inoculated plants.

Advantages and Disadvantages of Artificial Inoculation Methods

In all inoculation methods, a mycelial plug was used as the inoculum source to study Phomopsis stem canker development. The use of mycelial plugs as the inoculum source in greenhouse inoculations has several advantages. First and foremost, the mycelial inoculum is easy to prepare, and a few culture plates are sufficient to inoculate a large number of plants. Second, it is a more reliable way to ensure infection than in a field environment, which can be heavily influenced by abiotic factors such as rainfall, humidity, temperature, and interference from other diseases or insect damage. Third, it is cost effective and possible to inoculate sunflower plants with multiple isolates of *D. helianthi* (Encheva and Kiryakov 2002). Lastly, studies can be conducted multiple times throughout the year, whereas field screening is typically limited to one growing season.

Although greenhouse-based screening using mycelial plugs as the inoculum source has many advantages, it does not replicate the natural infection process of Phomopsis stem canker, which begins as ascospore infection on foliar tissue. Previous evaluations of sunflower resistance using artificial inoculation methods and in field nurseries under natural infection have generally produced consistent results, but not always. Encheva and Kiryakov (2002) demonstrated a significant positive and strong correlation ($r = 0.86$) between the disease severity observed on the sunflower plants in the greenhouse using a straw test method and infection under natural conditions, and they indicated that using mycelial plugs as the source of inoculum was nearly as effective as ascospores under natural conditions. Viguié et al. (1999) evaluated the reaction of sunflower hybrids to Phomopsis in field plots where infested sunflower residue was spread between rows. Results found that the correlation coefficient for hybrids evaluated between mycelium tests on leaves and in the field environment was significant and strong ($r = 0.73$). However, the correlation coefficient for inbred lines evaluated between the mycelium tests and under seminatural infection was only moderate ($r = 0.56$) and not significant (Viguié et al. 1999).

Conclusions and Recommendations

Results in this study suggests that all four inoculation methods evaluated may provide useful data for evaluating aggressiveness of pathogen isolates or, presumably, screening germplasm for resistance to Phomopsis stem canker (Mathew et al. 2018). However, symptomology and pathogen recovery results suggest that the stem-wound inoculation method may be the most user friendly. In addition, this method may be a cost- and time-effective way to help

screen germplasm for resistance to the disease. However, it should not replace the evaluation of elite germplasm for resistance to *Phomopsis* stem canker in natural field environments.

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