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Inoculation Method Impacts Symptom Development Associated with *Diaporthe aspalathi*, *D. caulivora*, and *D. longicolla* on Soybean (*Glycine max*)

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Abstract

One hundred fifty-two *Diaporthe* isolates were recovered from symptomatic soybean (*Glycine max*) stems sampled from the U.S. states of Iowa, Indiana, Kentucky, Michigan, and South Dakota. Using morphology and DNA sequencing, isolates were identified as *D. aspalathi* (8.6%), *D. caulivora* (24.3%), and *D. longicolla* (67.1%). Aggressiveness of five isolates each of the three pathogens was studied on cultivars Hawkeye (*D. caulivora* and *D. longicolla*) and Bragg (*D. aspalathi*) using toothpick, stem-wound, mycelium contact, and spore injection inoculation methods in the greenhouse. For *D. aspalathi*, methods significantly affected disease severity ($P < 0.001$) and pathogen recovery ($P < 0.001$). The relative treatment effects (RTE) of stem-wound and toothpick methods were significantly greater than for the other methods. For *D. caulivora* and *D. longicolla*, a significant isolate × method interaction affected disease severity ($P < 0.05$) and pathogen recovery ($P < 0.001$). Significant differences in RTEs were observed among *D. caulivora* and *D. longicolla* isolates only when the stem-wound and toothpick methods were used. Our study has determined that the stem-wound and toothpick methods are reliable to evaluate the three pathogens; however, the significant isolate × method interactions for *D. caulivora* and *D. longicolla* indicate that multiple isolates should also be considered for future pathogenicity studies.

Soybean (*Glycine max* (L.) Merr.) is one of the most important crops grown in the United States. In 2017, soybean production totaled 119.4 million metric tons (United States Department of Agriculture-National Agricultural Statistics Service [USDA-NASS] 2018; https://www.nass.usda.gov/), which resulted in $40.5 billion of revenue according to the 2017 market values. Among the production constraints of soybean in the United States, diseases caused by species of *Diaporthe* are important (Wrather et al. 1997). As of 2018, five *Diaporthe*-associated diseases have been reported in the United States, and these include: (1) pod and stem blight, caused by *Diaporthe sojae* Lehman (Lehman 1923; Udayanga et al. 2015) and *D. longicolla* (Hobbs) Santos, Vrandecic & Phillips (Cui et al. 2009; Hobbs et al. 1985; Mathew et al. 2015a; Santos et al. 2011; Udayanga et al. 2015); (2) northern stem canker caused by *D. caulivora* (Athow & Caldwell) Santos, Vrandecic & Phillips (Athow and Caldwell 1954; Santos et al. 2011); (3) southern stem canker caused by *D. aspalathi* Jansen, Castlebury & Crous (van Rensburg et al. 2006); (4) stem disease caused by *D. gulyae* Shivas, Thompson & Young (Mathew et al. 2018a); and (5) Phomopsis seed decay caused by *D. longicolla* (Hobbs et al. 1985; Sinclair 1993; Udayanga et al. 2015). The total estimated yield losses from the *Diaporthe*-associated diseases in the United States and Canada (Ontario) were approximately 1 million metric tons in 2014 (Allen et al. 2017). At this time, options to manage *Diaporthe*-associated diseases of soybean are limited. Fungicides (seed treatments, foliar) may be labeled for *Diaporthe*-associated diseases; however, their efficacy against the causal pathogens are unknown. Sources of resistance to *Diaporthe*-associated diseases have been identified (Chang et al. 2016; Chiesa et al. 2009; Keeling 1985; Kilen et al. 1985; Li et al. 2015; Pioli et al. 2003); but there is little known about the presence of resistance genes currently deployed in soybean cultivars available to farmers.

Over the past five decades, researchers have used numerous inoculation techniques in the greenhouse to study *Diaporthe* isolate aggressiveness and to screen soybean genotypes for resistance (Campbell 2016; Chen et al. 2009; Chiesa et al. 2009; Crall 1952; Keeling 1988, 1982; Kontz et al. 2016; Li et al. 2010; Lu et al. 2010; Mengistu et al. 2007; Pioli et al. 2003). For example, Keeling (1982) identified soybean genotypes with resistance to *D. caulivora*, while Kontz et al. (2016) identified resistance to both *D. caulivora* and *D. longicolla*. Both the researchers used the toothpick inoculation method for their experiments in the greenhouse. Pioli et al. (2003) compared the virulence of isolates of *D. aspalathi* and *D. caulivora* on soybean genotypes carrying four major loci (*Rdm1* to *Rdm4*) using the toothpick method and concluded that the *Rdm* genes that confer resistance to *D. aspalathi* do not provide resistance to *D. caulivora*. Mengistu et al. (2007) observed death of ‘Maverick’ when the hypocotyl of the plants was inoculated with *D. longicolla* isolates from pitted morning-glory (*Ipomoea lacunose* L.) and nodding spurge (*Euphorbia nutans* Lag.). Chen et al. (2009) observed symptoms caused by *D. sojae* isolates (brown lesions and pycnidia arranged in linear rows) on soybean plants, when the stems were injected with a conidial suspension of the isolates in the greenhouse. Li et al. (2010) observed significant differences in aggressiveness among *D. longicolla* isolates recovered from soybean and weed hosts, when plants of ‘Williams 82’ were inoculated with these isolates using the cut seedling assay under greenhouse conditions. Campbell (2016) optimized the toothpick inoculation method to

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assess virulence of *D. aspalathi* isolates on a stem canker susceptible cultivar (G81-2057) by testing various interactions of inoculation locations on soybean plants, wound sealant, and plant age at the time of inoculation in the greenhouse.

In addition to using several inoculation techniques to study *Diaporthe* on soybean in the greenhouse, researchers have used different methods to evaluate disease severity caused by these stem pathogens. For example, Li et al. (2010) measured stem length and lesion length to compare the aggressiveness of *D. longicolla* isolates from soybean and weed hosts on Williams 82. Lesion length has also been used to differentiate the level of resistance to *D. caulivora* in soybean (Keeling 1988; Thickett et al. 2007). Mengistu et al. (2007) recorded the number of dead seedlings of Maverick to study the effect of *D. longicolla* and other species of *Diaporthe* recovered from weeds on soybean. Pioli et al. (2003) used two methods to quantify disease severity caused by *D. aspalathi* and *D. caulivora* on soybean plants. The first method was a visual rating scale of 0, 0.5, and 1 with the numbers corresponding to the severity symptoms ranging from no lesion development on the plants to complete plant death. The second method was based on classifying the interaction between soybean and *Diaporthe* isolates as compatible or incompatible depending on the number of dead plants (Pioli et al. 2003). Other researchers have used either the visual rating scale of 0, 0.5, and 1 (Benavidez et al. 2010; Chiesa et al. 2009) or percentage of dead seedlings (Campbell 2016) to identify resistance to *D. aspalathi* and *D. caulivora* in soybean.

While several inoculation techniques and disease evaluation methods are available, very few studies have considered variation in isolate aggressiveness of the species of *Diaporthe* causing stem disease in different soybean producing regions of the United States. For example, Lu et al. (2010) compared the aggressiveness of nine *D. caulivora* isolates from Iowa and observed significant differences in the incubation period, lesion expansion rate, lesion length, and time to plant death among isolates. Li et al. (2010) compared the aggressiveness of 35 soybean isolates of *D. longicolla* from eight production areas of the United States and observed significant differences in stem length and lesion length among isolates. However, with the revisions to the naming of species affecting soybean within the *Diaporthe* genus (Santos et al. 2011; Udayanga et al. 2015; van Rensburg et al. 2006), it may be essential to revisit the aggressiveness of *Diaporthe* isolates collected from different geographical origins on soybean. Aggressiveness studies of *Diaporthe* isolates can help understand the pathogenic variability, and to select isolates for developing soybean varieties with broad-based resistance to these pathogens. The questions then arise on what inoculation technique and disease evaluation methods can be effectively used to screen the stem pathogens, *D. aspalathi*, *D. caulivora*, and *D. longicolla*, on soybean and if these inoculation techniques always require wounding to allow optimal infection by these pathogens. Furthermore, it may be important to know if the inoculation methods can affect symptoms and isolate aggressiveness of the three pathogens collected based on the geographic origin of the isolate. The specific objective of this study was to compare four commonly used inoculation methods to assess the aggressiveness of isolates of *D. aspalathi*, *D. caulivora*, and *D. longicolla* on a susceptible soybean cultivar in the greenhouse.

Materials and Methods

***Diaporthe* isolates collection and identification.** Prior to this study, 152 *Diaporthe* isolates were recovered from diseased plants sampled from commercial fields in Indiana (2014 to 2016), Iowa (2014 to 2016), Kentucky (2016), Michigan (2014), and South Dakota (2002 to 2007; 2014 to 2016) where symptoms, such as reddish-brown lesions or a canker, were observed along the stem or branches of the plants (Table 1). In each of the five states, at least five soybean fields were selected arbitrarily where *Diaporthe*-associated diseases were observed each year of the survey, and the fields were located more than 5 km from a previously selected field. In each soybean field, 10 plants exhibiting stem blight and/or stem canker symptoms were sampled at each of the two collection sites along five long transects (50 m). These transects were randomly selected in such a way that an area of approximately 0.4 ha was observed in each field while sampling soybean plants.

To isolate the causal pathogens from plants, the stems were rinsed in tap water for 3 min. The diseased stem of each plant was cut into small pieces (1 cm long). Stem pieces were surface-disinfested in 0.05% sodium hypochlorite for 1 min, 70% ethanol for 1 min, washed with sterile water, and then dried on filter paper. Three stem pieces from each plant were placed on potato dextrose agar (PDA; Oxoid, Basingstoke, UK) plates that were incubated at 22°C for 10 days under 12 h of alternating light/dark conditions. Cultures were examined using an Olympus CX31 Binocular Microscope (Olympus Corporation, Center Valley, PA) and scored for the presence of *Diaporthe* based on morphological characteristics (Santos et al. 2011; Udayanga et al. 2015; van Rensburg et al. 2006), which included colony appearance on PDA, production of fruiting bodies, and sporulation. After 10 days of growth, *Diaporthe* isolates were hyphal-tipped with a sterile scalpel from the leading edge of the original cultures and transferred to fresh plates of PDA. In addition, developing mycelia of the *Diaporthe* isolates or spores (ascospores or conidia) exuding from fruiting structures (perithecia or pycnidia) produced by the fungus were transferred to fresh PDA plates. Of the 152 isolates, 102 were suspected to be *D. longicolla*, 13 isolates *D. caulivora*, and

Table 1. Isolates used in this study, genes sequenced, and GenBank accessions

Isolates[a]	Year of isolation	Location[b]	Species identity[c]	GenBank accession numbers	
				ITS	EF1-α
DIA-026	2016	Hopkins County, KY	*D. aspalathi*	MG776307	MG776322
DIA-030	2016	Hopkins County, KY	*D. aspalathi*	MG776308	MG776323
DIA-022	2016	Ballard County, KY	*D. aspalathi*	MG776309	MG776324
DIA-043	2016	Daviess County, KY	*D. aspalathi*	MG776310	MG776325
DIA-007	2016	Breckinridge County, KY	*D. aspalathi*	MG776311	MG776326
DIA-046	2016	Buena Vista County, IA	*D. longicolla*	MG776312	MG776327
DIA-016	2016	Ballard County, KY	*D. longicolla*	MG776313	MG776328
DIA-056	2016	Knox County, IN	*D. longicolla*	MG776314	MG776329
DIA-086	2016	Clay County, SD	*D. longicolla*	MG776315	MG776330
DIA-063	2016	Wabash County, IN	*D. longicolla*	MG776316	MG776331
DIA-068	2016	Fulton County, IN	*D. caulivora*	MG776317	MG776332
SD-026	2002	Hamlin County, SD	*D. caulivora*	MG776318	MG776333
SD-027	2003	Hamlin County, SD	*D. caulivora*	MG776319	MG776334
SD-029	2007	Hamlin County, SD	*D. caulivora*	MG776320	MG776335
MI-009	2014	Ingham County, MI	*D. caulivora*	MG776321	MG776336

[a] Fifteen isolates were selected from a total of 152 *Diaporthe* isolates based on species identity and geographical location.
[b] IA = Iowa, IN = Indiana, KY = Kentucky, MI = Michigan, SD = South Dakota.
[c] Species identity was established using the Basic Local Alignment Search Tool Nucleotide (BLASTN) searches at GenBank.

37 isolates *D. aspalathi* based on morphology. Five representative isolates each of the three pathogens were selected based on geographic region (state or county within state) for molecular identification and for use in the pathogenicity experiments.

For molecular identification of the selected representative isolates of each of the three pathogens, the internal transcribed spacer (ITS) and translation elongation factor 1-alpha (EF1-α) gene regions were sequenced. Prior to DNA extraction, the mycelium of each isolate was scraped from the surface of the respective 10-day old PDA culture and lyophilized. DNA was extracted from the lyophilized mycelia using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI) and checked for quality using NanoDrop (Thermo Fisher Scientific, Waltham, MA). The ITS and EF1-α gene regions of the 15 isolates were amplified using ITS1/ITS4 primers (White et al. 1990) and EF1-728F/EF1-986R primers (Carbone and Kohn 1999), respectively. The polymerase chain reaction (PCR) for the two gene regions was performed in a 25.0-µl mixture containing 2.0 µl of fungal DNA (10 ng/µl), 0.75 µl forward primer (10.0 µM), 0.75 µl reverse primer (10.0 µM), 12.5 µl of 2× Taq PCR Master Mix containing Taq DNA Polymerase (Qiagen, Valencia, CA), and 9.0 µl of sterile nuclease-free water. The PCR cycle for ITS was set up at denaturation at 95°C for 30 s, annealing at 55°C for 50 s, and extension at 72°C for 1 min with a total of 39 cycles. The PCR cycle for EF1-α was set at denaturation at 95°C for 30 s, annealing at 58°C for 50 s, and extension at 72°C for 1 min with a total of 39 cycles. To confirm amplification, PCR product (5 µl) of the isolates was analyzed by agarose gel electrophoresis (2% agarose gel). The PCR products were sequenced (Functional Biosciences, Inc., Madison, WI) using the ITS1/ITS4 primers (White et al. 1990) and the EF1-728F/EF1-986R primers (Carbone and Kohn 1999). DNA sequences of the isolates were edited using BioEdit (v7.2.5; Hall 1999) and analyzed using the Basic Local Alignment Search Tool Nucleotide (BLASTN) searches at GenBank (National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/). The isolates were identified to *Diaporthe* species based on top BLASTN results of the sequences in GenBank (e-value $< e^{-10}$, highest score, and >95% similarity). The sequences of the isolates generated in this study are deposited in GenBank under the accession numbers MG776307 to MG776321 for the ITS sequences and MG776322 to MG776336 for the EF1-α sequences (Table 1).

Comparison of inoculation methods. To determine the aggressiveness of *Diaporthe* isolates on soybean, four inoculation methods commonly used for *Diaporthe*-associated pathogenicity studies in different plant species (e.g., *Glycine max*, *Helianthus annuus*, *Lupinus albus*) were compared. These included: the stem-wound method (Benavidez et al. 2010; Chiesa et al. 2009; Mathew et al. 2015a, b, 2018a, b); toothpick method (Backes et al. 2005; Campbell et al. 2017; Keeling 1982; Lu et al. 2010; Pioli et al. 2003; Ploetz and Shokes 1987); mycelium contact method (Thompson et al. 2011); and spore injection method (Chen et al. 2009; Kmetz et al. 1979).

For each of the four inoculation methods, five isolates representing *D. aspalathi*, *D. caulivora*, and *D. longicolla* were used to inoculate plants of susceptible soybean cultivars Bragg (for *D. aspalathi*) and Hawkeye (for *D. caulivora* and *D. longicolla*) in separate experiments (Table 1). Two seeds of Bragg or Hawkeye were planted into a 3.78-liter pot filled with a mixture of potting mix (Sunshine Mix-1, Sun Grow Horticulture Products, Belleview, WA), vermiculite (Vermiculite, PVP Industries, Inc., North Bloomfield, OH), and organic garden soil (Ecoscraps, Provo, UT) in a 4:1:1 ratio. For all three pathogens, the pots were maintained in a greenhouse (air temperatures of 27 ± 2°C for *D. caulivora* and *D. aspalathi*, and 24 ± 2°C for *D. longicolla*; relative humidity ≥90%; 12 h of alternating light and dark conditions) and watered once every other day.

To obtain inoculum, for the stem-wound, mycelium contact, and toothpick inoculation methods, *Diaporthe* isolates were grown on PDA at 22°C for 10 days under 12 h alternating light and dark conditions. To obtain inoculum for the spore injection method, the same 15 isolates were grown on PDA containing sterile toothpicks at 22°C for 25 days under 12 h of alternating light and dark conditions. For all inoculation methods, plants were inoculated at the second to third trifoliate growth stage of soybean (V2–V3) based on research by Campbell (2016) and Smith and Backman (1989).

For the stem-wound method, a wound was made ≈50 mm below the first trifoliate node on the stem of the soybean plants with the help of an autoclaved 200-µl pipette tip. A mycelial plug (≈5 mm diameter) was taken from the margin of a 10-day-old *Diaporthe* culture and placed into the wound. The wound was sealed with petroleum jelly (Vaseline, Unilever, Rotterdam, Netherlands) to avoid dehydration (Campbell 2016; Crall 1952). For noninoculated plants, a noninfested PDA plug was placed in the wound and sealed with petroleum jelly.

For the mycelium contact method, a mycelial plug (≈5 mm diameter) was placed in contact with the stem portion of the soybean plants ≈50 mm below the first trifoliate node. Petroleum jelly was applied over the top of the plug to avoid dehydration. For the noninoculated plants, inoculation was performed similarly using a noninfested PDA plug.

For the toothpick method, autoclaved wooden flat toothpicks (Diamond, Hearthmark, Rye, NY) were placed on PDA plates containing the *Diaporthe* culture of interest, and the plates were incubated at 22°C for 15 days under 12 h alternating light and dark conditions. After 15 days, when the toothpicks were colonized by the fungus, they were inserted into the stems of the soybean plants at an angle (≈45 degrees) and ≈50 mm below the first trifoliate node. A noninfested toothpick was inserted into the stems of the noninoculated plants. The inoculation site was sealed with petroleum jelly.

For the spore injection method, autoclaved flat toothpicks were placed on PDA plates containing the *Diaporthe* cultures. After 15 days, when the toothpicks were colonized by the fungus and covered with perithecia, they were placed in approximately 100 ml of sterile distilled water and macerated in an electric blender (Jarden Corporation, Rye, NY). The resulting suspension was filtered through cheesecloth to remove the lumps of mycelium, broken toothpicks, and culture medium. The type of spores in the suspension (ascospores of *D. caulivora* and *D. aspalathi* or α-conidia of *D. longicolla*) were confirmed using the Olympus CX31 Binocular Microscope. The spore count was determined by using a hemocytometer (VWR, Radnor, PA). Approximately 1 ml of the spore suspension containing 1×10^7 ascospores of *D. caulivora* or *D. aspalathi* and 1×10^7 α-conidia of *D. longicolla* was injected into soybean stems ≈50 mm below the first trifoliate node with the help of a disposable hypodermic syringe with a 22-gauge needle (3 ml). Before injecting, the spore suspension was forced through the needle tip to mix the inoculum and then reloaded into the disposable syringe. Sterile distilled water was injected into the stems of noninoculated plants. The site of injection of both inoculated and noninoculated plants was sealed with petroleum jelly.

For all inoculation methods, the plants were misted for 3 s every 5 min for 3 days after inoculation and then for 10 s every 3 h until the end of experiment (21 days postinoculation). At 21 days after inoculation, the disease severity was measured as 0 = plant showed no lesions; 0.5 = plant showed elongated lesions along the stem (length of the lesion >1 cm when compared with noninoculated plants) but no plant death; and 1 = plant dead (Fig. 1; Benavidez et al. 2010; Campbell 2016; Chiesa et al. 2009; Pioli et al. 2003).

In addition, the effectiveness of inoculation methods was assessed based on recovery of pathogen from the inoculated plants. To determine pathogen recovery, the stems of all plants were cut from the point of inoculation (2.54 cm above and below) into small pieces (≈2 cm length). The pieces were washed in distilled water for 2 min, surface-disinfested with 0.05% sodium hypochlorite and 70% ethanol for 1 min each, rinsed with sterile water, and then dried with sterile filter paper. The stem pieces were placed onto PDA plates amended with 0.02% streptomycin sulfate. The plates were incubated at 22°C for 7 to 14 days under 12 h of alternating light and dark conditions. The cultures were scored for the presence (isolation rating of '1') or absence (isolation rating of '0') of *Diaporthe* based on colony morphology.

Statistical analyses were performed separately for *D. aspalathi*, *D. caulivora*, and *D. longicolla*. For each pathogen, a completely

randomized design in factorial arrangement (four inoculation methods × five isolates) was established. For the analyses, inoculation methods and isolates were regarded as the experimental factors and each pot containing two plants was considered as the replication. The treatment combinations (inoculation method × isolate) were randomly assigned to experimental units (soybean plants) during the experiment. The experiment for each of the three pathogens was conducted three times with five replicate pots (total 10 plants; 2 plants per pot) per treatment combination. Since the disease rating data did not have a normal distribution, they were analyzed using the nonparametric procedure of Brunner et al. (2002) as described by Shah and Madden (2004). Before combining the data from the three experimental repeats for each pathogen, the data were first checked for homogeneity of variance using the Fligner-Killeen test in R (R core team 2013; https://www.rstudio.com/) at $P = 0.05$. Following the homogeneity of variance test, the main and interactive effect of inoculation method and isolate was determined using analysis of variance type-statistics (ATS) of ranked data in the nparLD package (Noguchi et al. 2012) in R. The Relative Treatment Effects (RTE) was calculated as probability means derived from the marginal distribution functions of ranked data using the equation: $RTE = (R – 0.5)/N$; where R is the mean rank of the treatments and N is the total number of observations (Singer et al. 2004). To compare treatments using RTE, confidence intervals (CI) were calculated using the nparLD package in R at $P = 0.05$.

In addition, the Pearson product-moment correlation coefficient (r) was calculated using the cor.test function in R for every combination of RTE of disease severity and pathogen recovery associated with the soybean plant in each experiment of *D. aspalathi*, *D. caulivora*, and *D. longicolla*. The noninoculated plants (inoculated with noninfested PDA plug for stem-wound method, mycelium contact method, and toothpick method, or water for spore injection method) did not show any symptoms, and no pathogens were recovered. Hence the disease rating and isolation rating data for the noninoculated control plants were not included in the statistical analyses.

Results

Diaporthe **isolates collection and identification.** For *D. aspalathi*, the 37 isolates produced dense white mycelium, scattered circular or irregular stroma, and globose perithecia on PDA. The ITS and EF1-α sequences of the five *D. aspalathi* isolates matched the type sequence of *D. aspalathi* strain CBS 117168 (Accession Numbers KC343035 and KC343761) with 99% identities and 0% gaps. For *D. caulivora*, the 13 isolates produced white to light brown colonies, circular and infrequent stromata, and spherical pycnidia (diameter up to 300 µm) on PDA. The ITS and EF1-α sequences of the five *D. caulivora* isolates matched the type sequence of *D. caulivora* strain CBS 127268 (Accession Numbers KC343046 and KC343771) with 100% identities and 0% gaps. For *D. longicolla*, the 102 isolates produced a white dense colony with characteristic yellowish ring and pycnidia with long necks on PDA. The ITS and EF1-α sequences of the five *D. longicolla* isolates matched the type sequence of *D. longicolla* isolate FAU601 (Accession Numbers KJ590729 and KJ590768) with 100% identities and 0% gaps.

Fig. 1. Soybean plants inoculated with the *Diaporthe longicolla* isolate DIA-056 (center) and *D. aspalathi* isolate DIA-030 (right) using the toothpick inoculation method in the greenhouse (Photo credit: Nathan Braun). Disease severity was measured at 21 days after inoculation as 0 = plant showed no lesions (left); 0.5 = plant showed elongated lesions (length of the lesion >1 cm when compared with noninoculated control plants), but no plant death (center); and 1 = plant dead (right) (Benavidez et al. 2010; Campbell 2016; Chiesa et al. 2009; Pioli et al. 2003).

Comparison of inoculation methods. As for symptom development, lesions of varying length (30 to 50 mm for *D. longicolla*, 60 to 80 mm for *D. aspalathi*, and 70 to 100 mm for *D. caulivora*), girdling of the stem, and plant death were observed when soybean plants of Hawkeye and Bragg were inoculated with either of the three pathogens using the toothpick and stem-wound inoculation methods. In contrast, only lesions of varying length (30 to 50 mm) were observed when plants were inoculated with the three pathogens using the mycelium contact and spore injection inoculation methods.

For the three pathogens, the Fligner-Killeen for homogeneity of variance test did not result in significant differences in the variance of the three experimental repeats for disease severity (*D. aspalathi* [$P = 0.192$], *D. caulivora* [$P = 0.083$], and *D. longicolla* [$P = 0.060$]) and pathogen recovery (*D. aspalathi* [$P = 0.055$], *D. caulivora* [$P = 0.680$], and *D. longicolla* [$P = 0.615$]). Hence the disease severity and pathogen recovery results of the three experiments were combined for additional nonparametric analyses.

Diaporthe aspalathi. There was a significant effect of inoculation methods on disease severity (ATS = 61.2; df = 2.88; $P = 3.50 \times 10^{-38}$) and pathogen recovery (ATS = 25.8; df = 2.20; $P = 96.44 \times 10^{-13}$). As for disease severity (expressed in terms of RTE), RTEs of the stem-wound and toothpick inoculation methods were significantly greater (based on 95% CI) than that of mycelium contact and spore injection inoculation methods. However, no significant differences in RTEs were observed between the stem-wound and toothpick inoculation methods or between the spore injection and mycelium contact inoculation methods (Table 2). For pathogen recovery (expressed in terms of RTE), RTEs of the stem-wound method and toothpick method were significantly greater (based on 95% CI) than that of mycelium contact and spore injection inoculation methods. However, no significant differences in RTEs were observed between the stem-wound and toothpick inoculation methods or between the spore injection and mycelium contact inoculation methods (Table 2). A significant correlation was observed between disease severity and pathogen recovery across the four inoculation methods ($r = 0.999$; $P = 0.0004$).

Diaporthe caulivora. A significant isolate × inoculation method interaction was observed to affect disease severity (ATS = 19.2; df = 5.29; $P= 3.65 \times 10^{-20}$) and pathogen recovery (ATS = 5.6; df = 5.90; $P = 9.36 \times 10^{-6}$). Disease severity of *D. caulivora* isolates varied significantly within inoculation methods. For the stem-wound method, RTE of the isolate SD-029 was significantly greater than that of DIA-068, MI-009, SD-026, and SD-027. The RTEs of DIA-068 and MI-009 were significantly greater than that of SD-026 and SD-027. No significant differences in RTEs were observed between SD-026 and SD-027 or between DIA-068 and MI-009. For the toothpick method, RTEs of isolates SD-029, DIA-068, and MI-009 were significantly greater than that of SD-026 and SD-027. No significant differences in RTEs were observed among SD-029, DIA-068, and MI-009 or between SD-026 and SD-027. For mycelium contact and spore injection inoculation methods, no significant differences in RTEs were observed among *D. caulivora* isolates (Table 3). Pathogen recovery of *D. caulivora* isolates varied significantly within inoculation methods. For the stem-wound inoculation method, RTEs of isolates SD-029, DIA-068, and MI-009 were significantly greater than that of SD-026 and SD-027. No significant differences in RTEs were observed among SD-029, DIA-068, and MI-009 or between isolates SD-026 and SD-027. For the toothpick inoculation method, RTEs of isolates SD-029, DIA-068, and MI-009 were significantly greater than that of SD-026 and SD-027. No significant differences in RTEs were observed among SD-029, DIA-068, and MI-009 or between SD-026 and SD-027. For mycelium contact and spore

injection inoculation methods, no significant differences in RTEs were observed among *D. caulivora* isolates (Table 3). A significant correlation was observed between disease severity and pathogen recovery across the four inoculation methods ($r = 0.978$; $P = 0.021$) and five isolates ($r = 0.984$; $P = 0.002$).

Diaporthe longicolla. A significant isolate × inoculation method interaction was observed to affect disease severity (ATS = 2.9; df = 6.17; $P = 0.007$) and pathogen recovery (ATS = 2.1; df = 6.45; $P = 0.0460$). Disease severity of *D. longicolla* isolates varied significantly within inoculation methods. For the stem-wound method, RTEs of the isolates DIA-056 and DIA-086 were significantly greater than that of DIA-063, DIA-045, and DIA-016. No significant differences in RTEs were observed between isolates DIA-086 and DIA-056 or among isolates DIA-063, DIA-016, and DIA-045. For the toothpick method, the RTE of isolate DIA-063 was significantly greater than that of the isolate DIA-016. In addition, no significant differences in RTEs were observed among isolates DIA-045, DIA-086, DIA-056, and DIA-016. For mycelium contact and spore injection inoculation methods, no significant differences in RTEs were observed among *D. longicolla* isolates (Table 4). Pathogen recovery of *D. longicolla* isolates varied significantly within inoculation method. For the stem-wound inoculation method, RTE of isolate DIA-086 was significantly greater than that of DIA-016 and DIA-045; and RTE of DIA-056 was significantly greater than that of DIA-016. No significant differences in RTEs were observed among isolates DIA-086, DIA-063, and DIA-056; or among isolates DIA-016, DIA-045, and DIA-063. For the toothpick inoculation method, RTE of isolate DIA-063 was significantly greater than that of isolate DIA-016. No significant differences in RTEs were observed among DIA-016, DIA-045, DIA-056, and DIA-086. For mycelium contact and spore injection inoculation methods, no significant differences in RTEs were observed among *D. longicolla* isolates (Table 4). A significant correlation was observed between disease severity and pathogen recovery across the four inoculation methods ($r = 0.998$; $P = 0.001$) and five isolates ($r = 0.986$; $P = 0.002$).

Table 2. Mean rank, relative treatment effects, and confidence interval of relative treatment effects to determine the effect of inoculation methods on disease severity and pathogen recovery for *Diaporthe aspalathi* in the greenhouse

	Disease severity		Pathogen recovery	
Inoculation methods	**Mean rank**	**Relative treatment effect[a,b]**	**Mean rank**	**Relative treatment effect[b,c]**
Stem wound	279.3	0.69 (0.66, 0.72) *	242.0	0.60 (0.57, 0.63) *
Toothpick	282.9	0.70 (0.67, 0.73) *	246.0	0.61 (0.58, 0.64) *
Mycelium contact	137.0	0.34 (0.31, 0.27)	168.0	0.42 (0.37, 0.46)
Spore injection	102.6	0.25 (0.22, 0.28)	146.0	0.36 (0.32, 0.40)

[a] Disease severity caused by *D. aspalathi* on cv. Bragg was assessed at 21 days after inoculation as 0 = plant showed no lesions; 0.5 = plant showed elongated lesions along the stem (length of the lesion >1 cm when compared with noninoculated plants), but no plant death; and 1 = plant dead (Campbell 2016; Pioli et al. 2003).

[b] Statistical analyses for disease severity and pathogen recovery were performed separately. For the two analyses, the Relative Treatment Effect (RTE) was calculated using the equation: RTE = (R – 0.5)/N; where R is the mean rank of the treatments and N is the total number of observations (Singer et al. 2004). Confidence intervals (in parentheses) were calculated to compare RTEs at $P \leq 0.05$.

[c] *D. aspalathi* was isolated from the inoculated plants on PDA and identified by morphology (van Rensburg et al. 2006).

Table 3. Mean rank, relative treatment effects, and confidence interval of relative treatment effects to determine the effect of inoculation methods on disease severity and pathogen recovery for *Diaporthe caulivora* in the greenhouse

		Disease severity		Pathogen recovery	
Inoculation methods	**Isolates**	**Mean rank**	**Relative treatment effect[a,b]**	**Mean rank**	**Relative treatment effect[b,c]**
Stem wound	DIA-068	312.3	0.77 (0.70, 0.84) *	314.5	0.78 (0.72, 0.83) *
	MI-009	320.5	0.80 (0.71, 0.86) *	294.5	0.73 (0.64, 0.80) *
	SD-026	172.4	0.42 (0.36, 0.50)	194.5	0.48 (0.38, 0.59)
	SD-027	190.1	0.47 (0.39, 0.56)	194.5	0.48 (0.38, 0.59)
	SD-029	360.6	0.90 (0.87, 0.92) *	324.5	0.81 (0.79, 0.82) *
Toothpick	DIA-068	289.2	0.72 (0.63, 0.80) *	314.5	0.78 (0.72, 0.83) *
	MI-009	242.4	0.60 (0.50, 0.70) *	274.5	0.68 (0.57, 0.77) *
	SD-026	154.8	0.38 (0.34, 0.43)	194.5	0.48 (0.38, 0.58)
	SD-027	154.8	0.38 (0.34, 0.43)	184.5	0.46 (0.36, 0.56)
	SD-029	326.0	0.81 (0.70, 0.89) *	314.5	0.78 (0.73, 0.82) *
Mycelium contact	DIA-068	146.0	0.36 (0.35, 0.37)	134.5	0.33 (0.28, 0.38)
	MI-009	146.0	0.36 (0.35, 0.37)	134.5	0.36 (0.29, 0.42)
	SD-026	146.0	0.36 (0.35, 0.37)	134.5	0.33 (0.29, 0.38)
	SD-027	163.6	0.40 (0.35, 0.47)	144.5	0.36 (0.29, 0.42)
	SD-029	146.0	0.36 (0.35, 0.37)	134.5	0.33 (0.28, 0.38)
Spore injection	DIA-068	146.0	0.36 (0.35, 0.37)	134.5	0.33 (0.29, 0.38)
	MI-009	146.0	0.36 (0.35, 0.37)	154.5	0.38 (0.31, 0.46)
	SD-026	146.0	0.36 (0.35, 0.37)	134.5	0.33 (0.28. 0.38)
	SD-027	146.0	0.36 (0.35, 0.37)	134.5	0.33 (0.29, 0.38)
	SD-029	154.8	0.38 (0.34, 0.43)	154.5	0.38 (0.30, 0.46)

[a] Disease severity caused by *D. caulivora* on cv. Hawkeye was assessed at 21 days after inoculation as 0 = plant showed no lesions; 0.5 = plant showed elongated lesions along the stem (length of the lesion >1 cm when compared with noninoculated plants), but no plant death; and 1 = plant dead (Campbell 2016; Pioli et al. 2003).

[b] Statistical analyses for disease severity and pathogen recovery were performed separately. For the two analyses, the Relative Treatment Effect (RTE) was calculated using the equation: RTE = (R – 0.5)/N; where R is the mean rank of the treatments and N is the total number of observations (Singer et al. 2004). Confidence intervals (in parentheses) were calculated to compare RTEs at $P \leq 0.05$.

[c] *D. caulivora* was isolated from the inoculated plants on PDA and identified by morphology (Santos et al. 2011).

Discussion

The study presented here suggests that the inoculation methods have a significant impact on symptom development caused by *D. aspalathi*, *D. caulivora*, and *D. longicolla* on soybean in greenhouse inoculations. For *D. aspalathi*, inoculating plants with the toothpick and stem-wound inoculation methods resulted in the greatest disease severity, with symptoms including lesions of varying length (60 to 80 mm), girdling of the stem, and plant death. For *D. caulivora* and *D. longicolla*, plants inoculated with the stem-wound and toothpick inoculation methods produced significant differences in disease severity among isolates, and the symptoms observed included lesions of varying length (70 to 100 mm for *D. caulivora* and 30 to 50 mm for *D. longicolla*) and plant death. In contrast to the toothpick and stem-wound methods, plants inoculated with the mycelium contact and spore injection methods resulted in lower disease severity (only 30 to 50 mm long lesions) among inoculation methods (for *D. aspalathi*) or among isolates (for *D. caulivora* and *D. longicolla*). The four inoculation methods were further assessed by recovery of pathogen from the inoculated plants. For *D. aspalathi*, *D. caulivora*, and *D. longicolla*, a significant and strong correlation was observed between disease severity and pathogen recovery across the four inoculation methods, indicating the recovery of the three pathogens also varied by inoculation method. The pathogen recovery for *D. aspalathi* was greatest from plants inoculated with the stem-wound and toothpick methods. For *D. caulivora* and *D. longicolla*, pathogen recovery varied significantly among isolates for the stem-wound and toothpick methods. From the plants inoculated with the mycelium contact and spore injection methods, recovery of all the three pathogens was relatively lower. The greater recovery of *D. aspalathi*, *D. caulivora*, and *D. longicolla* supports the reliability of toothpick and stem-wound inoculation methods to evaluate isolate aggressiveness of the three pathogens on soybean in the greenhouse.

Among the inoculation methods used in this study, the toothpick method is commonly used to study aggressiveness and virulence of *Diaporthe* isolates on soybean (e.g., Campbell et al. 2017; Crall 1952; Keeling 1982; Lu et al. 2010; Pioli et al. 2003). In most studies, *Diaporthe*-infested toothpicks are inserted into the base of the stem of the soybean plants (Crall 1952; Keeling 1982; Pioli et al. 2003) and the inoculations were made on soybean plants when they are 10 to 14 days old (Keeling 1982; Pioli et al. 2003). However, in our study, we performed toothpick inoculations between the unifoliate and trifoliate of the soybean plants, and this was because Crall (1952) observed that regardless of the level of resistance in soybean varieties, plants inoculated in the base of the stem using infested toothpicks were mostly killed. Even so, we observed the toothpick inoculation method to be advantageous to study isolate aggressiveness of *Diaporthe* on soybean. First, in this study, significant differences in disease severity and pathogen recovery were observed among *D. caulivora* and *D. longicolla* isolates at 21 days after inoculation. For *D. aspalathi*, significantly greater disease severity was produced on soybean plants when the toothpick inoculation method was used. Second, this technique provides an ideal system to deliver inoculum for pathogens that infect soybean through wounded tissue. Third, in this study, recovery of *D. aspalathi*, *D. caulivora*, and *D. longicolla* was highest when the toothpick inoculation method was used. Despite the advantages of using the toothpick inoculation method, including the ease of use and limited or no influence by environment based on the study by Keeling (1988), Keeling (1985) reported conflicting disease phenotyping results when this technique was used. For instance, 7% of the susceptible cv. J77-339and 39% of the susceptible Bragg were observed to be resistant when the plants were inoculated with *D. caulivora* using the toothpick inoculation method (Keeling 1985). However, our research did not examine the effectiveness of the toothpick and other inoculation methods to assess the levels of resistance to the three pathogens, and this must be considered in a future study.

Compared with the toothpick inoculation method, the stem-wound method has been used in the last decade to evaluate species of *Diaporthe* on soybean. For example, Chiesa et al. (2009) used the stem-wound method to screen populations derived from a cross of J77-339 (*rdm/rdm*) × Hutcheson (*Rdm4/Rdm4*) to *D. aspalathi* and identified *Rdm* loci (*Rdm4* and *Rdm5*), which conferred specific resistance to two physiological races of the pathogen (CE109 and CE112).

Table 4. Mean rank, relative treatment effects, and confidence interval of relative treatment effects to determine the effect of inoculation methods on disease severity and pathogen recovery for *Diaporthe longicolla* in the greenhouse

		Disease severity		Pathogen recovery	
Inoculation methods	Isolates	Mean rank	Relative treatment effect[a,b]	Mean rank	Relative treatment effect[b,c]
Stem wound	DIA-016	223.4	0.55 (0.44, 0.66)	222.0	0.55 (0.44, 0.66)
	DIA-045	253.6	0.63 (0.52, 0.72)	252.0	0.62 (0.52, 0.72)
	DIA-056	326.6	0.81 (0.74, 0.86) *	302.0	0.75 (0.70, 0.79) *
	DIA-063	292.3	0.72 (0.63, 0.80)	282.0	0.70 (0.61, 0.77)
	DIA-086	322.3	0.80 (0.73, 0.85) *	312.0	0.77 (0.76, 0.79) *
Toothpick	DIA-016	197.6	0.49 (0.38, 0.60)	202.0	0.50 (0.39, 0.61)
	DIA-045	227.8	0.56 (0.47, 0.65)	232.0	0.57 (0.47, 0.67)
	DIA-056	275.1	0.68 (0.59, 0.76)	282.0	0.70 (0.61, 0.77)
	DIA-063	288.0	0.71 (0.62, 0.79) *	282.0	0.70 (0.62, 0.76) *
	DIA-086	266.5	0.66 (0.55, 0.75)	262.0	0.65 (0.55, 0.74)
Mycelium contact	DIA-016	115.5	0.28 (0.26, 0.30)	122.0	0.30 (0.25, 0.35)
	DIA-045	141.4	0.35 (0.29, 0.41)	152.0	0.37 (0.30, 0.46)
	DIA-056	115.5	0.28 (0.26, 0.30)	132.0	0.32 (0.26, 0.39)
	DIA-063	124.1	0.30 (0.27, 0.34)	122.0	0.30 (0.26, 0.34)
	DIA-086	124.1	0.30 (0.26, 0.36)	122.0	0.30 (0.25, 0.36)
Spore injection	DIA-016	141.4	0.35 (0.29, 0.41)	142.0	0.35 (0.28, 0.43)
	DIA-045	132.8	0.33 (0.27, 0.39)	132.0	0.32 (0.26, 0.39)
	DIA-056	150.1	0.37 (0.29, 0.45)	162.0	0.40 (0.31, 0.50)
	DIA-063	132.8	0.33 (0.27, 0.38)	132.0	0.32 (0.26, 0.39)
	DIA-086	158.7	0.39 (0.31, 0.48)	162.0	0.40 (0.31, 0.50)

[a] Disease severity caused by *D. longicolla* on cv. Hawkeye was assessed at 21 days after inoculation as 0 = plant showed no lesions; 0.5 = plant showed elongated lesions along the stem (length of the lesion >1 cm when compared with noninoculated plants), but no plant death; and 1 = plant dead (Campbell 2016; Pioli et al. 2003).

[b] Statistical analyses for disease severity and pathogen recovery were performed separately. For the two analyses, the Relative Treatment Effect (RTE) was calculated using the equation: RTE = (R – 0.5)/N; where R is the mean rank of the treatments and N is the total number of observations (Singer et al. 2004). Confidence intervals (in parentheses) were calculated to compare RTEs at $P \leq 0.05$.

[c] *D. longicolla* was isolated from the inoculated plants on PDA and identified by morphology (Udayanga et al. 2015).

Mathew et al. (2018a) used the stem wound method to compare the symptoms caused by *D. caulivora* with *D. gulyae* on inoculated soybean plants. In this study, an advantage to using stem-wound technique was that it is effective in detecting significant differences in disease severity and pathogen recovery among *D. caulivora* and *D. longicolla* isolates. For *D. aspalathi*, greater disease severity and pathogen recovery was produced on plants inoculated with the stem-wound inoculation method, which was not significantly different from the toothpick method. Another important advantage to using the stem-wound inoculation method is the higher pathogen recovery based on this research and the study by Mathew et al. (2018b). The other benefits of the stem-wound inoculation method as observed by Denman and Sadie (2001) were efficient use of space, time required to inoculate plants, as well as labor and cost associated with the greenhouse inoculations. However, Denman and Sadie (2001) observed the need to standardize the stem-wound inoculation method to reduce variation in lesion length caused by the causal pathogen among genotypes and within genotypes to differentiate susceptible and resistant cultivars. In this study, standardization of the stem-wound method and other inoculation methods to study the isolate aggressiveness was not considered and must be examined in future.

Besides the toothpick and stem-wound inoculation techniques, two less invasive inoculation methods—spore injection and mycelium contact—were tested for their effectiveness to study *Diaporthe* isolate aggressiveness. The less invasive inoculation methods have three prime advantages. First, the methods involve nonaggressive wounding of plants. Second, the resistance due to structural barriers that prevents infection by the disease causing pathogen is included when the germplasm is evaluated (Chen and Wang 2005). Third, these techniques may be as effective as the wounding methods to study *Diaporthe* on soybean; however, the disease may take longer to develop. For example, Thompson et al. (2011) used the wound inoculation method and mycelium contact inoculation method to study the pathogenicity of three fungi (*D. gulyae*, *D. kochmanii* Shivas, Thompson & Young [syn. *D. sojae*], and *D. kongii* Shivas, Thompson & Young) causing Phomopsis stem canker of sunflower (*Helianthus annuus* L.). It was observed that although the Phomopsis stem canker severity was similar between the two inoculation methods, disease symptoms (tan colored lesion or cankers) were observed on the sunflower plants inoculated with the mycelium contact method 1 to 7 days later than the wounding method (Thompson et al. 2011). In this study, disease severity was significantly lower for *D. aspalathi* or among isolates for *D. caulivora* and *D. longicolla* when the soybean plants were inoculated with the mycelium contact or spore injection inoculation methods, and this may be because of two reasons. First, the infection of soybean by *Diaporthe* requires wounding. This was speculated by Cowley et al. (2012), who used the spore injection inoculation method to evaluate resistance in *Lupinus albus* L. to *D. toxica* Williamson, Highet, Gams & Sivasithamparam. Cowley et al. (2012) did not identify resistance to *D. toxica* among genotypes and they speculated that *L. albus* genotypes were susceptible to the pathogen only if the infection coincides with wounding of the genotypes from hail or insect feeding. Second, species of *Diaporthe* exhibit a longer incubation period; for instance, a study by Rupe et al. (1999) showed that for disease development by *D. aspalathi* on soybean, an incubation period of at least 34 to 41 days is required before the symptoms are observed on the plants. In our study, the incubation period for the three pathogens was only 21 days postinoculation for all inoculation methods, which was possibly shorter for the pathogens to establish and cause disease as observed when the toothpick and stem-wound inoculation methods were used. We also suspect that the shorter incubation period may have affected pathogen recovery in this study. However, there is also the possibility that the *Diaporthe* isolation data were affected because of contaminations with other fungi (e.g., *Alternaria*) on PDA, which was mostly observed when plants inoculated using the spore injection and mycelium contact inoculation methods were placed on culture media.

In summary, based on the results of our study, the toothpick and stem-wound inoculation methods are effective techniques to assess the aggressiveness of isolates of *D. aspalathi*, *D. caulivora*, and *D. longicolla* on soybean. However, the significant interactions between inoculation methods and isolates for *D. caulivora* and *D. longicolla* indicate that multiple individual inoculations using single isolates or mixtures of isolates should also be considered for pathogenicity studies in future. Between the two techniques, the toothpick inoculation method is more commonly used to screen *Diaporthe* on soybean because Keeling (1982) observed that the response of soybean genotypes to ascospore infection by *D. caulivora* in the field was similar to that when the plants were inoculated with *D. caulivora*-infested toothpick in the greenhouse. However, there are no studies comparing greenhouse and field phenotyping results for *D. aspalathi* and *D. longicolla* using the toothpick inoculation method, and this must be examined in the future.

In general, greenhouse screening for *Diaporthe* resistance in soybean has the benefits of being less time-consuming and less affected by environmental conditions (e.g., temperature, humidity) when compared with screening in the field. However, the success of screening in the greenhouse will depend on the plant age, host genetics, quality and quantity of fungal inoculum, inoculation method, and environmental conditions. While few of these factors, such as plant age (Campbell 2016; Rupe et al. 1999; Smith and Backman 1989; Thickett et al. 2007), host genetics (Chiesa et al. 2009; Keeling 1985, 1982; Kilen et al. 1985; Li et al. 2015; Pioli et al. 2003; Thickett et al. 2007), and quality of the fungal inoculum (Keeling 1982; Kmetz et al. 1979), have been explored to study species of *Diaporthe* (e.g., *D. aspalathi*, *D. caulivora*, *D. longicolla*) for their virulence on soybean, more research related to standardization of inoculation methods, quantity of the fungal inoculum, and environmental conditions is warranted for all soybean pathogens in the *Diaporthe* genus.

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