Isolation and pathogenicity of *Phomopsis* from symptomless sunflower

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Introduction

Phomopsis stem canker is a major disease of sunflower that cause severe yield losses in the United States (Mathew et al. 2015). To date, three species of *Diaporthe*: *D. helianthi* Muntañola-Cvetkovic, Mihaljcevic, and Petrov, *D. gulyae* Shivas, Thompson, and Young (Mathew et al. 2015) and *D. stewartii* Harrison (Olson et al. 2017) have been reported in the United States. Among the causal pathogens, *D. helianthi* overwinter as perithecia in crop debris. In spring, when the perithecia mature, globular to cylindrical asci are produced, which mature at optimum temperatures (15 to 30°C) and release ascospores (Androsova et al. 2008). These ascospores are splashed by rain or carried by wind to infect leaves of the sunflower plants. Following leaf infection, the fungus proceeds to infect the petiole and then stem of the plants (Mathew et al. 2018). After 20 to 25 days of leaf stem, lesions become visible on the stem when the plant reaches the flowering stage (Mathew et al. 2018).

Recent studies related to management options (e.g. fungicides) in the U.S. have shown inconsistent efficacy results (F. Mathew, *unpublished*). One of the hypothesis behind inconsistency in the efficacy results is that species of *Diaporthe* may be "latent" in sunflower as described in other crops, such as soybean, *Glycine max* L. (Kmetz et al. 1979). Kmetz et al. (1979) observed that *Diaporthe phaseolorum* var. *sojae* can cause latent infection of immature soybean tissues, which includes cotyledons, hypocotyls, stems, petioles, and pods. Thus, the current study was conducted to determine the species composition and pathogenicity of the endophytic fungi belonging to the genus *Diaporthe* isolated from sunflower.

Materials and methods

1. Isolation of species of *Diaporthe*

Field experiments were conducted in South Dakota, North Dakota and Nebraska at one location each in 2019. In each location, large plots (50 ft length by 10 ft width) were planted. A hybrid susceptible to Phomopsis stem canker (e.g. Rh 400 CL (CHS genetics) in South Dakota), was used. Plants were scored for presence of Phomopsis stem canker on a biweekly basis (between V2 (two true leaves) and R5 growth stage (flowering stage) of sunflower development) using a 0-5 rating scale (Mathew et al. 2015). Additionally, three to five plant samples were taken at each scoring, cut into parts (stems, leaves, and roots) and air-dried. At the time of sampling, growth stage of sunflower development was recorded.

For fungal isolation, stems of the sunflower plants were chopped into pieces and six pieces of stem tissue were randomly selected. The stem pieces were surface-sterilized in ethanol (70%) and sodium hypochlorite (10%) for 30 seconds each and placed on potato dextrose agar (PDA) media. After incubation at room temperature (~25ºC) for two to five days, fungal colonies were transferred to fresh PDA plates. The isolates were identified to species level based on morphology and qPCR assays (Elverson et al. 2020). The recovery of fungi belonging to the *Diaporthe* genus was calculated in percentage by the following formula

$$Diaporthe\ recovery\ percentage\ (\%) = \frac{Number\ of\ Diaporthe\ isolates}{6} \times 100$$

2. Pathogenicity test of isolates of *Diaporthe*

Following isolation and identity confirmation, seven isolates of *Diaporthe* were randomly selected and evaluated for their pathogenicity on a susceptible sunflower hybrid [Rh 400 CL (CHS genetics)] in the greenhouse (Table 1). The experiment was set up in a completely randomized design with six replications (pot containing two plants each).

Table 1. List of *Diaporthe* isolates tested for pathogenicity in sunflower in the greenhouse

S.N.	Treatments	Species	Growth stage of the sunflower plants during sampling from the field trial
1	N-58	*D. gulyae*	R1-R2
2	N-31	*D. longicolla*	R3-R4
3	N-4	*D. gulyae*	R5-R6
4	N-1	*D. helianthi*	R5-R6
5	N-3	*D. helianthi*	R5-R6
6	N-5	*D. helianthi*	R5-R6
7	N-6	*D. helianthi*	R5-R6
8	Non-inoculated control	PDA plug	

The isolates of *Diaporthe* were inoculated at R1 stage of sunflower development (the terminal bud forms a miniature floral head rather than a cluster of leaves; Berglund 2007). The stem wound method (Mathew et al. 2015) was used for inoculation where stem was wounded at internode around 30 cm above ground level by micropipette tip and a 7 day old mycelial plug (4 mm) was inserted into the wounded area. Similarly, a PDA plug (without the fungus) was inserted into the wounded area for non-treated control. The pots were placed in a greenhouse maintained at temperatures between 20ºC and 25ºC with a 16 h photo period (light intensity was about 450 $\mu Em^{-2}s^{-1}$). For all inoculated plants, the wounded area was covered with petroleum jelly. The inoculated plants were misted for two minutes for every two hours for three days. After three days of misting, the plants were watered every other day. Fifteen days post-inoculation, disease severity was rated

using a 0 to 5 scale (Mathew et al. 2015). Data was analyzed using non-parametric statistics in RStudio 1.2 (https://rstudio.com/)

Results

1. Isolation of species of *Diaporthe*.

In the field trials, sunflower plants did not show any symptoms till V5-V6 (five to six true leaf) stage. Brown-colored lesions was observed on few lower leaves at R1-R2 stage (miniature terminal bud formation to bud size less than one inch), on few petioles at R3-R4 (bud size more than one inch to bud open with ray floret open), and on leaves, petioles and stems at R5-R6 stage (flowering). However, *D. longicolla* and *D. gulyae* were isolated from stem of all growth stages except V2-V4 (two to four true leaf) (Figure 1). *Diaporthe helianthi* was recovered from stems sampled at R5-R6 growth stage of the crop development. This indicates that the isolates of *Diaporthe* can be recovered from sunflower stems although there was no visible disease symptoms on the plants.

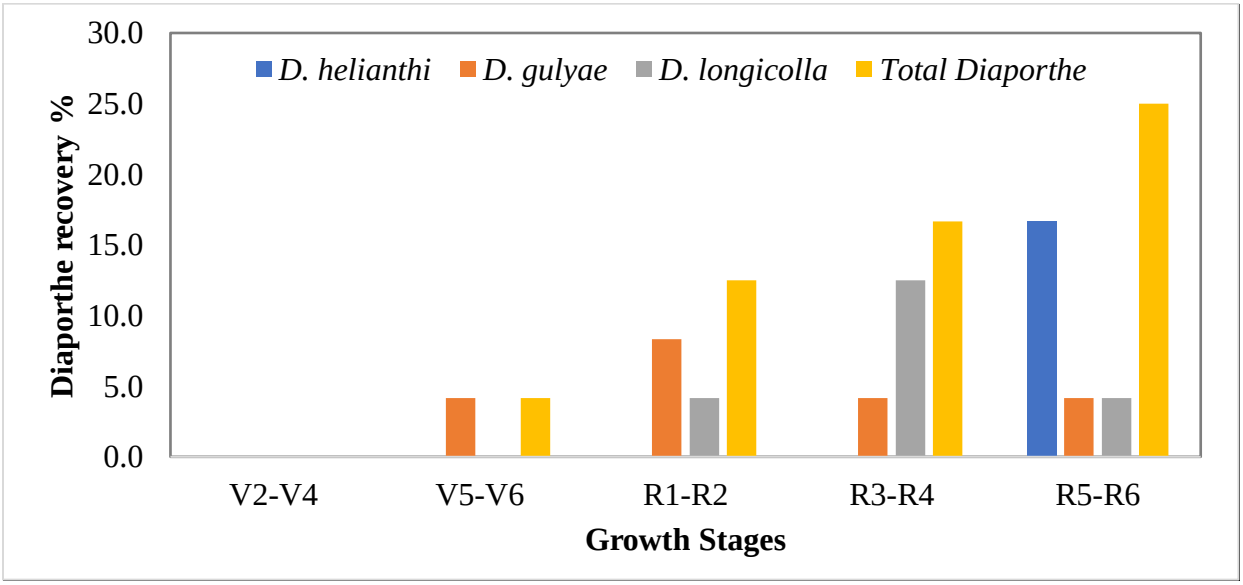

Figure 1. *Diaporthe* recovery percentage from samples collected at different growth stages of sunflower from the field trial in South Dakota (Growth stages: V2 – V4 = two to four true leaves, V5-V6 = five to six true leaves, R1-R2 = miniature terminal bud formation to bud size less than one inch, R3-R4 = bud size more than one inch to bud open with ray floret open, and R5-R6 = flowering.

2. Pathogenicity test of isolates of *Diaporthe*

Pathogenicity test revealed that all isolates of *Diaporthe* were pathogenic to sunflower. Isolates of *D. gulyae* and *D. helianthi* caused significantly higher disease severity (expressed as relative treatment effects) than the non-inoculated control and the isolate of *D. longicolla*.

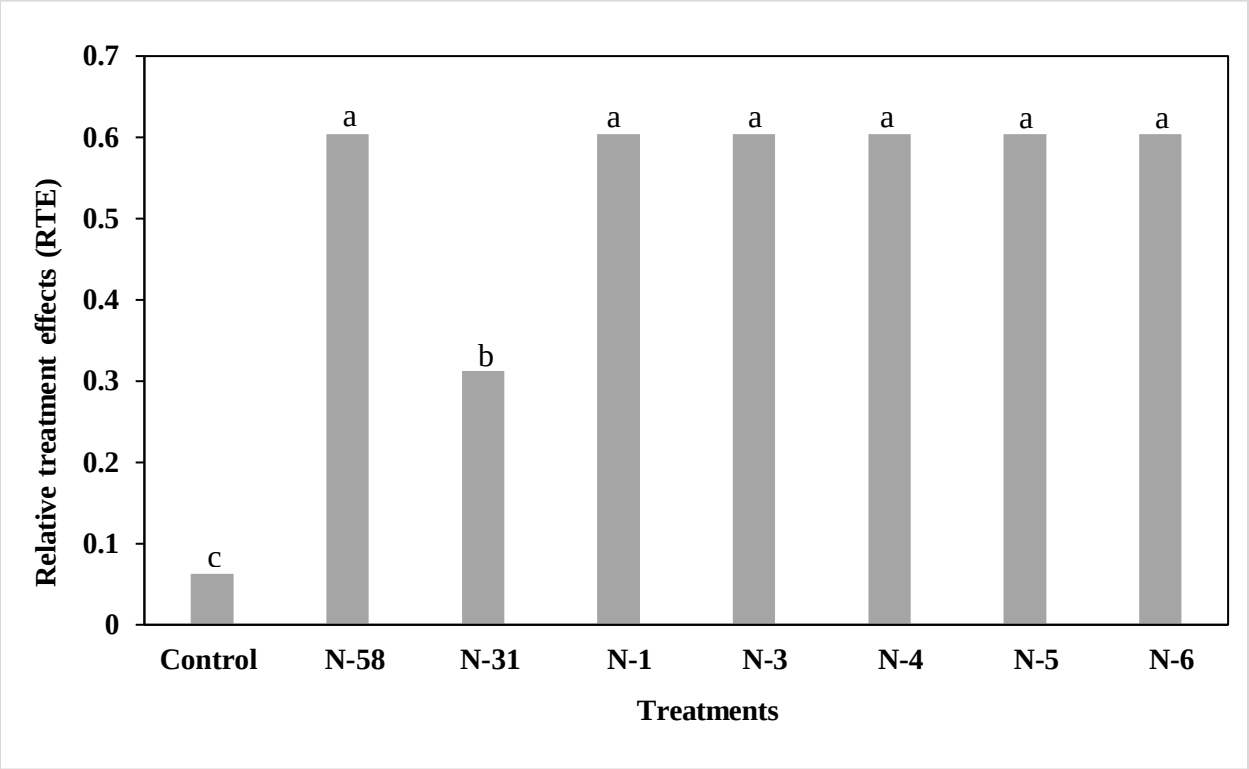

Figure 2. Disease severity (expressed as relative treatment effects) caused by isolates of *Diaporthe* on sunflower in the greenhouse (Control = Non-inoculated control; N-58, N-4 = *D. gulyae*, N-31= *D. longicolla;* and N-1, N-2, N-5, N-6= *D. helianthi*)

Future work

Isolation of *Diaporthe* from samples collected in North Dakota and Nebraska is in progress and will be completed by April 2020. Additionally, this field research will be repeated in 2020 at a single location in Nebraska, North Dakota and South Dakota.

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