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Development of southern stem canker disease on soybean seedlings in the greenhouse using a modified toothpick inoculation assay

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ABSTRACT

Southern soybean stem canker, caused by *Diaporthe aspalathi,* has caused major soybean losses for growers in the Southeast U.S. The most effective disease management tool for growers is the use of stem canker resistant soybean varieties. A fast, reliable greenhouse assay for stem canker would facilitate identification of resistance in soybean germplasm. An existing toothpick assay was modified to include culturing *D. aspalathi* on oxgall agar on toothpicks autoclaved in oxgall liquid medium. Three week-old seedlings were inoculated with toothpicks inserted in the stem between the cotyledon and the first trifoliate leaf. Inoculation sites were sealed with petroleum jelly, and seedlings were incubated in humidity chambers for 72 h. Stem canker disease was highly consistent on susceptible lines four weeks post-inoculation and was not observed on soybean lines with known *Rdm* genes. High levels of disease (≥98.3%) were observed with cultivars Braxton, Davis, and Centennial previously reported to have resistance in field studies. Isolates of *D. aspalathi* were observed to differ in virulence. This modified greenhouse assay will assist in the efforts for breeding stem canker resistance and better understanding the differences in disease phenotypes for some cultivars.

1. Introduction

Southern stem canker of soybean, caused by *Diaporthe aspalathi* (Syn. *Diaporthe phaseolorum* var. *meridionalis*), inflicted losses of approximately 854,000 metric tons from 1980 to 89 in the southeastern United States (Wrather et al., 1995). In severe cases, field losses reached levels of up to 80% (Krausz and Fortnum, 1983). Decreased use of susceptible cultivars has since reduced incidence of southern stem canker in the U.S. (Ploetz et al., 1986). Losses of 40,635 tons were reported in 15 southern U.S. states in 2005 (Wrather and Koenning, 2006). Southern soybean stem canker has also been a significant problem for South American soybean growers. In 1998, Argentina experienced losses of 1.28 million metric tons, and Brazil and Bolivia had losses of 10,000 metric tons, respectively (Wrather et al., 2001).

Diaporthe aspalathi is an ascomycetous fungus that overwinters on soybean crop debris, infecting the subsequent crop by rain splashing of ascospores or conidia onto wounded stems and petioles (Backman et al., 1985). Infection typically takes place early in the spring during vegetative growth stages with symptoms appearing much later during reproductive stages of the plants (Fehr et al., 1971). Symptoms include development of reddish-brown lesions on or in the stem, which may lengthen along the stem and become sunken and necrotic on susceptible varieties. Leaves on infected plants may develop interveinal chlorosis and can eventually become necrotic, remaining attached to the stem (Backman et al., 1985). The infected vascular tissue blocks the translocation of water to the seed-bearing portion of the plant, resulting in reduced yield and plant death in severe cases (Hildebrand, 1952).

Southern stem canker can be managed through cultural practices such as tilling or removing crop debris, weed control, and use of proper planting densities (Rothrock et al., 1985; Freitas et al., 2002). Systemic foliar fungicides applied early in the season can be effective against stem canker when applied to cultivars of intermediate susceptibility (Weaver et al., 1988). However, the most effective disease management tool is the use of soybean varieties with resistance to *D. aspalathi* in conjunction with the burial of inoculum by tillage (Backman et al., 1985). Host resistance is conditioned by at least five single, dominant genes designated as

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Rdm1, *Rdm2* (Kilen and Hartwig, 1987), *Rdm3*, *Rdm4* (Bowers et al., 1993), and *Rdm5* (Chisea et al., 2009; Pioli et al., 2003).

Most greenhouse evaluations of stem canker resistance have used a toothpick inoculation method first described by Crall (1952) and then modified by Keeling (1982) and Ploetz and Shokes (1989). *D. aspalathi*-colonized toothpicks are inserted into soybean stems to initiate disease. Keeling's toothpick colonization was conducted in liquid growth medium (Keeling, 1982), while Ploetz and Shokes (1989) utilized toothpicks colonized on growth medium amended with agar. The toothpick inoculation method has been used with varied success. Keeling (1982) observed a mix of susceptible and resistant plants in the resistant cultivars Tracy and CNS and the susceptible cultivar J77-339. Bowers et al. (1993) observed 12.5% of resistant Dowling seedlings susceptible to stem canker using the toothpick inoculation and attributed this to mixed seed. Four percent of susceptible 'Johnston' soybean plants were observed to be resistant (Bowers et al., 1993). Kilen and Hartwig (1987) reported one resistant out of 18 J77-339 seedling after stem inoculations. In contrast, Tyler (1996) observed consistent disease reactions of two resistant and four susceptible (including J77-339) soybean genotypes using Keeling's toothpick inoculation procedure. Weaver et al. (1988) concluded that the toothpick inoculation technique could be an alternative to field tests due to the unreliability of stem canker symptom expression in the field. Ideally, there should be a consistent disease phenotype on plants of the same genotype when inoculated with a common pathogen isolate.

The objectives of this study were to determine the effect of wound sealant, plant growth stage at time of inoculation, inoculation location, and growth media (recipe and presence of agar) on subsequent development of stem canker symptoms on susceptible soybean genotype G81-2057 using a toothpick inoculation in the greenhouse. The modified inoculation procedure was then tested with three isolates of *D. aspalathi* on a panel of 23 soybean cultivars/lines and also used to assess the virulence of seven isolates of *D. aspalathi* on eight susceptible soybean cultivars/lines.

2. Materials and methods

2.1. Diaporthe aspalathi isolates

During the fall season of 2013–2015, stems from susceptible soybean cultivar/line Hutton and G81-2057 exhibiting stem canker symptoms were collected from the UGA soybean stem canker nurseries in Bledsoe and Calhoun, Georgia. Stem sections (30–40 cm long) were collected from symptomatic plants. Stems were cut into ~5 cm pieces, surface sterilized for one min in 70% ethanol, followed by two min in 1% sodium hypochlorite, removed from the sodium hypochlorite and blotted dry. Individual pieces were placed onto acidified potato dextrose agar (A-PDA). Putative *D. aspalathi* isolates growing from the stem sections were transferred to fresh A-PDA. Five isolates (CA10-13, CA13-13, BL39-14, BL22-14, BL3-15; CA designates isolate collected at Calhoun, GA, BL designates isolates collected at Bledsoe, GA) with the morphological characteristics of *D. aspalathi* were subsampled by transfer of hyphal tips onto A-PDA. Isolates had white to grey, tan or white hyphal growth with or without pycnidia development (van Rensburg et al., 2006). Two isolates of *D. aspalathi* (NA-16 and NB-16) were isolated from infected soybean supplied by the stem canker screening program of the University of Arkansas. The identity of all putative *D. aspalathi* isolates were verified by sequencing the ITS1 region of DNA using the primer pair ITS1 and ITS4 (Bertini et al., 1999). DNA was extracted using the Chelex method (Walsh et al., 2013). A small amount of hyphae was added to 300 mL of 10% Chelex solution, vortexed, centrifuged for 15 s, and then incubated for 20 min at 95 °C. Solutions were vortexed, centrifuged for 15 s and DNA recovered from the supernatant liquid. DNA concentration was diluted to 50 ng μL^{-1}. Samples were heated at 94 °C for two min and 40 cycles were then performed at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Samples were then held at 72 °C for five min. PCR products were checked for amplification and correct amplicon size (500–600 bp) on a 1% agarose electrophoresis gel. Amplified products were purified using ExoSAPit reagent (Affymetrix, Santa Clara, CA) and sequenced at the Georgia Genomics Facility using an Applied Biosystems 3730xl 96-capillary DNA Analyzer (Thermo Fisher Scientific, Waltham, MA). Sequences were entered into the NCBI website blastn suite using the standard nucleotide blast search. Positive identifications were based on ≥99% identity (0.0 E-value) with multiple *D. phaseolorum* var. *meridionalis* sequences. Sequences were deposited in Genbank (KY767002-KY767008). The 'Type' strain of *D. aspalathi* (*D. phaseolorum* var. *meridionalis* DPM 1F; ATCC 200236), originally isolated in Calhoun, Georgia (Fernández and Hanlin, 1996), was acquired from the American Type Culture Collection.

Long-term storage of isolates was done by lyophilizing *D. aspalathi*-infected stem tissues and short-term storage was on PDA slants at 4 °C at UGA plant pathology facilities in Griffin, GA. Fresh, working inoculum was maintained by subsampling isolates onto new PDA every two to three weeks.

2.2. Plant materials

The southern stem canker susceptible soybean line G81-2057 was utilized in the inoculation tests. G81-2057 was highly susceptible to *D. aspalathi* CA13-13 in preliminary experiments and has been used as a susceptible check in UGA stem canker field nurseries since the 1980s. Seeds were planted in Jolly Gardener Pro-Line C/B growing mix (Atlanta, GA) in 10 cm × 10 cm Kord Presto (Riverhead, NY) sheet pots arranged on the 12 border cells of 15 cell flats. The three middle cells were left vacant for better light penetration (Harris et al., 2015). Three seeds were planted in each pot and seedlings were thinned to two seedlings per pot 7–10 days after planting. All seedlings, except those to be used in the plant age study, were allowed to grow for three weeks (12 h supplemented light) before inoculation. Seedlings were supported with 36-inch stakes two weeks after planting to prevent stems from snapping due to the mechanical damage caused by the toothpick inoculation method. Seedlings were watered three to four times a week and fertilized once a week with a Dosmatic model A30 dispenser (Hydro Systems Co., Carrollton, TX) set to deliver 200 $\mu g\ mL^{-1}$ N from a stock of 20-20-20 Scotts Peters water soluble fertilizer.

2.3. Toothpick inoculation assay

Soybean seedlings were inoculated with *D. aspalathi* by inserting fungus-colonized toothpicks into the stem of the plants. The toothpick assay described by Keeling (1982) and modified by Ploetz and Shokes (1989) was followed in the initial experiments. Briefly, round toothpicks were cut in half and autoclaved twice in fresh distilled water. Twenty toothpick halves were placed in a radial pattern on the surface of potato dextrose agar (PDA) in 9-cm petri plates. Plates were seeded in the center of the toothpicks with a mycelial plug of *D. aspalathi* CA13-13 grown for two weeks on PDA. Plates were sealed with parafilm and incubated at room temperature (~22 °C) on a laboratory bench for three weeks to allow the fungus to colonize the toothpicks. A small puncture wound was made in the stem above the cotyledon with a dissecting needle and the *Diaporthe*-colonized toothpick half was inserted to inoculate each seedling. All experiments included a non-colonized toothpick as a negative control.

Three experiments were conducted to determine the effects of: