IMPROVEMENT OF SOUTHERN STEM CANKER SCREENING METHODS AND CHARACTERIZATION OF GEORGIAN *DIAPORHTE ASPALATHI* ISOLATES

by

MARY ALLISON CAMPBELL

(Under the Direction of Zenglu Li and James Buck)

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 help ensure elite germplasm is resistant to this disease. An existing toothpick assay was modified to include culturing *D. aspalathi* on oxgall agar on toothpicks pre-soaked in oxgall liquid medium. Inoculation was performed at growth stage V2 between cotyledons and the first trifoliate, 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 and was not observed on resistant germplasm. More than 99.0% disease incidence was observed across three isolates of *D. aspalathi* after 4 weeks. This improved greenhouse assay will assist in future breeding efforts for stem canker.

INDEX WORDS: soybean, southern soybean stem canker, *Diaporthe aspalathi,* virulence, isolate.

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Bachelor of Science, Mississippi State University, 2013

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2016

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December 2016

DEDICATION

I would like to thank my wonderful, supportive family for their patience and kind words of encouragement through my academic career. Their encouragement has made this research possible. This thesis is dedicated to them and everyone involved in agriculture and agriculture research.

ACKNOWLEDGEMENTS

I would like to acknowledge my advisors, Dr. James Buck and Dr. Zenglu Li, my committee members, Dr. Marin Brewer and Dr. Alfredo Espinoza-Martinez, Jerry Davis. the members of the UGA Soybean Breeding and Molecular Genetics Lab, and all the members of the UGA Soybean Pathology Lab in Griffin for all of their assistance and guidance in conducting this research.

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Chapter 1

LITERATURE REVIEW OF SOUTHERN SOYBEAN STEM CANKER

Soybean

Soybean (*Glycine max* (L.) Merrill) is one of the most important cultivated legumes in the U.S. and worldwide. In 2015, the total value of soybean in the U.S. was approximately $34.5 billion with 3.93 billion bushels cultivated, which was a slight increase from 3.92 billion bushels produced in 2014. This was achieved across production acreage of 82.7 million acres, a slight decrease from 2014. The U.S. led world soybean production in 2015 with 33% of production followed by Brazil at 31% of production. Forty-three percent of the total U.S. soybean production or 1.69 billion bushels were exported by the U.S. in 2015 (Soy Stats 2015, http://www.soystats.com; verified 6 November 2016).

There are many uses of soybean, but these products can be consolidated into two main product types: oil and meal. Soybean oil is used in products such as meat analogs, margarine, shortening, cooking oil, and salad dressings and has accounted for 57% of the U.S.'s vegetable oil (Soy Stats 2015, http://www.soystats.com; verified 6 November 2016) (Calhoon, 2003). A small and diminishing amount of soybean oil is also used in industrial products such as paints, varnishes, and resin products. Approximately 4% of soybean oil produced in the U.S. is used in these industrial products (Calhoon, 2003).

Soybean meal is used primarily in animal and poultry feeds. The average caloric protein in soybean is 41-48%, making it an excellent source of nutrition for animals (Liu, 1997).

History of Soybean

Soybean (*Glycine max* L. Merr.) is a leguminous crop that originated in China. Many diverse types of *Glycine soja,* an ancient relative of modern *Glycine max*, are found extensively throughout China, which supports the theory of a Chinese origin for soybean (Qui and Chang, 2010). The earliest written records of soybean cultivation are also of Chinese origin. The name for soybean in Chinese characters ('Shu') appears in several ancient manuscripts, some approximately 4,500 years old (Qui and Chang, 2010). Domestication most likely took place during the Shang dynasty approximately 1700-1100 B.C. or earlier.

In 1765, the Surveyor General of the Colony of Georgia, Henry Yonge, planted soybeans on his plantation in Thunderbolt, Georgia. This was at the request of Samuel Bowen, a former employee of the East India Company, who had acquired seed from China via London. Additionally, in 1770, Benjamin Franklin sent soybean seed from London to be planted by botanist John Bartram. By 1851, soybean had been introduced in Illinois and was then disseminated through the U.S. Corn Belt (Hymowitz, 1990).

Soybean Physiology

Soybean is a dichotomous, leguminous plant with an annual growth habit. Plants exhibit an erect growth, branching habit and typically reach a height of 75-125 cm by maturity (Shibles et al., 1974). The first leaves are unifoliate and opposite one another; all subsequent leaves are trifoliate and alternate along the stem (Sun, 1957).

Soybean is a diploid [2n=40] that exhibits a monoecous reproductive system. Female and male gametes are produced on the same gametophyte or flower. Thus, most fertilization and subsequent reproduction naturally occurs as a of result self-pollination. Flowers are produced terminally and in each of the leaf axils. Flowering and developmental habits may be determinate or indeterminate (Williams, 1950). If pollinated, an inflorescence will produce 2-20 pods containing 1-5 seeds per pod (Kato et al., 1954).

Soybean flowering is controlled by photoperiod and temperature sensitivity Carlson, 1973). The maturity group of the plant also has an effect on the soybean planting area of adaptation and timeline of maturity. Flowering is typically initiated within 6-8 weeks post emergence if the cultivar is grown within its area of adaptation. Flowering may continue for three over five weeks (Borthwick and Parker, 1938). Ten to fourteen days after flowering, pods are usually visible. Maturity is reached 50-80 days after flowering contingent on environmental factors (Carlson, 1973).

Soybean Breeding

Soybean yield and production in the U.S. has been on a steady rise over the last century. Much of the success of this crop can be attributed to successful soybean breeding (KeShun, 1997). As with most agronomic crops, there are numerous breeding goals to improve the soybean. Most of these objectives, such as disease and pest resistance, contribute to the overall goal of increased seed yield. One of non-yield goals is to increase oleic oil seed content that improves oxidative stability that in turn, greatly increases oil functionality and shelf life. In breeding populations of most oil crops, the correlation between seed yield and oil content has been proven to be positive.

Conversely, the correlation between protein content increase and seed yield is negative (Norman, 1978). This connection between seed yield and oil content warrants consideration by breeders pursuing other goals, especially that of seed yield.

Soybean diseases caused by fungi, bacteria, viruses, and nematodes cause significant yield loss each year. In 2009, there was an estimated yield reduction of 494 million bushels in soybean due to disease (Estimates of soybean yield reductions caused by diseases in the United States; verified 6 November 2016). This makes resistance to economically significant diseases a highly desired trait in commercial soybean varieties. Development of disease resistance varieties can help reduce these losses as well as minimize chemical applications used to manage pests.

Southern Stem Canker of Soybean

Southern soybean stem canker, caused by the fungus *Diaporthe aspalathi* (E. Jansen) (syn *D. phaseolorum* var *meridionalis*), is an economically damaging disease affecting soybean across the southern U.S. as well as globally (Backman et al., 1985). *D. aspalathi* infects the vascular system of soybean plants, inhibiting the translocation of water and nutrients. Infection results in reduced yield and even plant death in highly susceptible soybean cultivars (Hildebrand, 1952). In 1994, seven of the top 10 soybean producing countries reported significant losses due to soybean stem canker (northern and southern combined). In 1994, the U.S. suffered the greatest loss at 1,990,000 metric tons, followed closely by Brazil at a loss of 1,800,000 metric tons (Wrather et al., 1997). Other top soybean producing countries that suffered notable losses from stem canker in 1994 included Argentina, Bolivia, Canada, Italy, and Paraguay (Wrather et al., 1997). In 1998, losses were reduced overall, but the top 10 soybean producing countries still collectively

lost 191,100 metric tons to stem canker (Wrather et al., 2001). An average yield loss of 22,000 metric tons was recorded for the years 1990-1994 throughout the 16 southern states alone in the U.S. (Wrather et al., 1995). It is estimated that in 2014, northern and southern soybean stem canker caused a combined loss of almost 12.7 million bushels of soybean yield in the U.S. (Estimates of soybean yield reductions caused by diseases in the United States; verified 6 November 2016).

Pathogen History in the U.S.

During the 1950s, soybean stem canker came to prevalence and was recognized as one disease with one causal agent, *Diaporthe phaseolorum* (Backman et al., 1985). The soybean cultivars Blackhawk and Hawkeye were susceptible to this disease and were grown throughout the northern U.S., resulting in significant losses to stem canker during this period (Hildebrand, 1952). Farmers began planting soybean varieties resistant to stem canker, significantly decreasing the impact of this disease by the late 1950's (Weaver et. al., 1984). However, growers started to report losses to stem canker in southern U.S. states during the 1970s and 1980s. Mississippi first reported stem canker in 1973; Alabama in 1977; Tennessee in 1981; South Carolina and Georgia in 1982; Florida, Louisiana, and Arkansas in 1977; and Texas in 1984 (Backman et al., 1985). Reactions of cultivars across states varied greatly, which has made pinpointing the southern genesis of this disease epidemic nearly impossible. Southern soybean stem canker came to prevalence while the susceptible soybean cultivar Bragg was being grown widely in the southeast U.S, though the origin of this disease cannot be traced directly back to this (Backman et al., 1985).

Losses to soybean stem canker reached a peak in the southern states during the 1980's. In 1983, it was an estimated that yield loss was equivalent to $37 million due to southern soybean stem canker with some infestations reaching levels of 80% (Backman et al., 1985, Krarusz and Fortnum, 1983).

***Diaporthe aspalathi* Taxonomy**

The fungus that causes southern soybean stem canker*, Diaporthe aspalathi*, is a member of the Diaporthe/Phomopsis complex. The fungi in this complex account for many other significantly plant diseases worldwide including cankers, diebacks, root rots, fruit rots, leaf spots, blights, decay and wilts on a wide range of hosts (Santos et al., 2011). The sexually reproducing state of the stem canker causal agent was originally identified as *Diaporthe phaseolorum* var *sojae* (Lehman) Wehm (Welch and Gilman, 1948). *Diaporthe phaseolorum* var *sojae* was later found to actually be the causal agent of soybean seed decay. The asexually reproducing state was designated as *Phomopsis phaseoli* (Morgan-Jones, 1989). Welch and Gilman (1948) distinguished stem canker from pod and stem blight, but erroneously suggested that the causal agent of soybean stem canker was *D. phaseolorum* var. *batatis* (Harter & Field). Athow and Caldwell (1954) identified the causal agent and gave it varietal distinction as *Diaporthe phaseolorum* (Cke. & Ell.) Sacc. var *caulivora* Athow & Caldwell. Kulik (1984) suggested that a formae speciales distinction of the strains of *D. phaseolorum* capable of causing stem canker would be more appropriate. Backman et al. (1985) recognized distinctions between the stem canker causing isolates in the northern and southern U.S. This resulted in the southern and northern biotypes of stem canker receiving their own formae speciales designations of *D. phaseolorum* f.sp. *meridionalis* and *D. phaseolorum*

f.sp. *caulivora,* respectively (Morgan-Jones, 1989). Disease reports in the southern U.S. described noticeably more aggressive and heat tolerant soybean stem canker than observed in the northern soybean growing areas, further justifying this division (Backman et al., 1985).

The advent of DNA sequencing technology further refined the differences between the southern and northern stem canker pathogens. Random amplified polymorphic DNA (RAPD) analyses and phylogenetic analyses of the internal transcribed spacer (ITS) and elongation factor 1 (EF1) provided evidence that these varieties are actually different species (Zhang et al., 1998). In 2011, Santos et al. (2011) designated *Diaporthe phaseolorum* f.sp. *caulivora* as the species *Diaporthe caulivora.* Rensburg et al (2006) renamed *Diaporthe phaseolorum* f.sp. *meridionalis* as the species *Diaporthe aspalathi.*

D. aspalathi is an ascomycetous fungus. It reproduces asexually by alpha and beta conidia produced in pycnidia with a short or no pycnidial beak (Zhang et. al., 1998). During sexual reproduction, *D. aspalathi* produces ascospores in perithecia. The northern soybean stem canker causal agent, *D. caulivora* is morphologically distinguished by the absence of pycnidia. In culture on acidified potato glucose agar, *D. aspalathi* produces white-tanned colonies with tufted or rope-like mycelia, stroma remained undefined and perithecia occur solitarily (Pioli et al., 2003).

Use of morphological characteristics alone to identify members of the Diaporthe complex is unreliable. Some growth structures and patterns occur as a result of environment and condition of the host. DNA sequencing, particularly of the EF1-α and

ITS regions, provides a more reliable confirmation of the pathogen's identity (Fernández and Hanlin, 1996; Lu et al., 2009)**.**

It should be noted that numerous studies in which the stem canker pathogen isolated in southern states is referred to as *D. phaseolorum* var. *caulivora* (Table 1.1). Both northern and southern pathogens were once referred to as *D. phaseolorum* var. *caulivora,* so it could be assumed that these older studies were actually conducted using *D. aspalathi* if molecular confirmation of the pathogen identity was used and the fungal isolate was collected in the southern U.S. or South America.

Disease Cycle of *Diaporthe aspalathi*

D. aspalathi, along with other pathogens in the Diaporthe/Phomopsis complex, survives and overwinters mostly on infested soybean debris left in the field after harvest (Backman et al., 1985). In spring, conidia (asexual spores) and ascospores (sexual spores) are produced on this debris in their respective structures, pycnidia and perithecia. Both types of spores are capable of causing infection (Backman et al., 1985). Spread of inoculum occurs when water splash from irrigation or rain propels spores of *D. aspalathi* from crop debris to stems of a current crop. In the presence of moisture, spores can germinate and penetrate the plant in 4 to 18 hours. Older, shaded, or damaged tissues such as leaf scars are especially vulnerable to infection (Frosheiser, 1957). Once the fungus penetrates outer stem tissues, it colonizes the vascular tissue of the plant over the following weeks. This colonization inhibits the translocation of water and nutrients throughout the plant (Hildebrand, 1952).

Symptoms/Infection Mode

Southern stem canker first manifests as small, reddish-brown lesions extending from leaf scars, wounded tissue, or leaf nodes during early reproductive stages of the soybean. These lesions spread from more mature tissues to younger tissues in the apex of stems. As infection progresses and the plant reaches growth stage R5, the affected tissue transforms into sunken, necrotic cankers (Smith and Backman, 1989). These cankers usually remain unilateral (on one side of the affected stem), but girdling of whole stems by growth stage R5 (pod fill) has been observed (Smith and Backman, 1989). Highly susceptible soybean cultivars may die due to this vascular blockage (Lalitha et al., 1988).

During infection, *D. aspalathi* produces a phytotoxin, which leads to the vivid interveinal chlorosis characteristic of southern soybean stem canker. The toxin was first isolated from symptomatic soybean tissue by Lalitha et al. (1989). These plants had been inoculated using single-spore isolates of *D. aspalathi.* They observed a positive correlation of R^2=0.6 between disease severity and toxin concentration. It was also observed that each single spore isolate produced different amounts of the phytotoxin.

Virulence of *D. aspalathi*

Differences in virulence among isolates of *D. aspalathi* has been reported. Keeling (1985) observed significant differences in lesion length and plant death caused by isolates of *Diaporthe phaseolorum* var. *caulivora* (*D. aspalathi*) collected from Mississippi, Tennessee, and Ohio. Isolate 81-102, collected from Tennessee, caused lesion length similar to that caused by several of the Mississippi isolates, but did not kill plants. Isolate D0048M1, collected from Ohio, was essentially avirulent, causing an average lesion length of 1 cm and no plant death. There was also great variability in the

lesion lengths and plant death produced by the Mississippi isolates. Ploetz and Shokes (1989) further supported this by reporting significant differences in stem canker symptoms among vegetative compatibility groups when tested across various levels of stem canker resistance. Pioli et al. (2003) reported several of their *D. aspalathi* isolates caused disease in plants possessing *Rdm* genes, potentially invalidating the claim that one of the *Rdm* genes can condition resistance to all isolates of *D. aspalathi.* It was observed in their study that the eight *D. aspalathi* isolates caused plant death at different rates in one susceptible soybean line and multiple lines containing one or more *Rdm* genes.

Host-Pathogen Interaction

In the soybean-*D. aspalathi* host-pathogen interaction, the host response is possibly initiated through the lipoxygenase pathway. Leaves from both resistant and susceptible soybean cultivars exhibited higher levels of lipoxygenase specific activity than non-inoculated controls (Silva et al., 2001).

Phytoalexins potentially play a role in incompatible reactions between host and *D. aspalathi* effectors. In cases of southern soybean stem canker resistance, the stem develops a red lesion around the point of infection and subsequent phytoalexin accumulation. A more intense the color red indicates higher levels of the corresponding phytoalexins (Modolo et al., 2002). Stem discoloration is typically displayed during pod fill or growth stage R5 (Fehr et al., 1971). This reaction is indicative of the accumulation of certain glyceollin precursors that often follows exposure to abiotic and biotic stresses. Glyceollin precursors that produce this red coloration include glycinol and the isoprenylated compounds glyceolidin I and II (Ingham et al., 1998; Zähringer et al., 1981). The compound NO acts as a radical donor in conjunction with other signaling

molecules to initiate the conversation of daidzein into glyceollins, shown to be synchronized in response to treatment of soybean tissue with *D. aspalathi* elicitors (Modolo et al., 2002).

Factors Affecting Disease Severity

Factors that can contribute to the occurrence and severity of soybean stem canker include soybean growth stage, wetting period, tillage regime and insect pests. The growth stage at which a plant is exposed to *D. aspalathi* inoculum is crucial in soybean stem canker development. Rupe et al. (1999) measured disease incidence with inoculation dates correlating with V1, V4, V6, V10, and R2 growth stages of the plants. It was observed that stem canker symptoms manifested quickest with an inoculation date at the V6 growth stage of the plant. Disease took the longest to appear with inoculation taking place in the V1 growth stage. Plants typically displayed foliar symptoms only after the R2 (flowering) growth stage of the plant regardless of inoculation date in both repetitions of the study. However, foliar disease onset was delayed till R5 if inoculation occurred in the R2 stage of plant growth (Rupe et al., 1999).

Wetting periods for the proliferation of *D. aspalathi* can affect stem canker incidence (Damicone et al., 1987). A continuous wetting treatment over 144 hours and a discontinuous wetting treatment over 96 hours yielded the highest pathogen recovery seven days following inoculation. Plants that received no surface moisture yielded no disease (Damicone et al., 1987).

Tillage methods have also been shown to have significant effect on the persistence of *D. aspalathi* (Rothrock et al., 1985). Burying *D. aspalathi* infested plant residue reduces the chances for inoculum to be spread to the current crop. Use of a no-

tillage cropping system led to an increase from 2.2% disease instance to one of 84.4% the following year (Rothrock et al., 1985). Over a two year period, the percent of infection was consistently lower in a conventionally tilled field compared to a field that with no tillage treatment (Rothrock et al., 1985). Conversely, a study performed in the Cerrado region of Brazil found that disease incidence and severity were consistently lower in no-till areas compared to moderate-till areas (Freitas et al., 2002).

The presence of certain insect pests can also exacerbate the effect of *D. aspalathi* on soybean. Three cornered alfalfa hopper girdles the stems of soybean through feeding. These girdles can cause yield loss through plant death, suppression of bean development, or by causing lodging and breakage. These wounds can also allow for increased infection by *D. aspalathi*, longer canker lesions as well as yield reduction (Russin and Bothel, 1986).

Management of Soybean Stem Canker

Cultural practices can minimize losses caused by *D. aspalathi*. Adjusting plant density has been shown to have an effect on the incidence and severity of soybean stem canker (Rothrock et al., 1985). Dense planting schemes create a shaded and humid environment conducive to fungal growth and infection. Freitas et al. (2002) observed that at growth stage R 5.5, a planting density of 36 plants/m resulted in high incidence and high severity of stem canker on the susceptible soybean cultivar Cristalina. In the moderately resistant soybean cultivar, FT-101, disease severity and incidence were lowest at 8 plants/m. The resistant cultivar, FT-104, exhibited the lowest and statistically similar incidence and severity of disease at a density of 8, 15, and 21 plants/m compared to 36 plants/m (Freitas et al., 2002).

Planting date also has an effect on the severity of southern soybean stem canker (Weaver et al., 1984). The initial spread of inoculum usually occurs in May and early June (Backman, 1985), so planting during late June or early July can reduce the impact of this disease. However, late planting may have a negative effect on yield (Weaver et al., 1984).

Crop rotation every one to two years can also help in maintaining low disease levels (Rothrock et al., 1985). Crops selected for rotation schemes with soybean crops should be a non-host to pod and stem canker, stem blight, and *Phomopsis* seed decay fungi. A study conducted in Argentina identified sunflower and its crop residues as potential alternative hosts to southern soybean stem canker (de Alcaraz et al., 1998). Cotton has also been documented as an asymptomatic alternate host and a source of *D. aspalathi* inoculum (Roy and Miller, 1983). Corn, small grains, alfalfa, or forage grasses are also suitable non-hosts to use in rotations in the southeast U.S. (Rothrock et al., 1985).

Sanitation of farm equipment aids to prevent the spread of soybean stem canker from field-to-field. Any equipment used in a recently infested field or a field with disease history of stem canker should be carefully cleaned of any plant debris and soil prior to use in a new location. Even if plants have shown resistance to soybean stem canker in years after disease, planters should take caution (Backman et al., 1985).

Fungicides have been shown to provide disease control in soybean cultivars of intermediate susceptibility to soybean stem canker (Weaver et al., 1984). However, the return on investment for fungicide applications on resistant and fully susceptible cultivars is negligible. Systemic foliar fungicides, such as Benlate (active ingredient [a.i.]

benomyl; no longer in use), applied early in the growing season can impede active spore production and subsequent disease spread (Backman et al., 1985). Fungicide applications should be performed early in vegetative growth as spores are being actively produced by *D. aspalathi* on plant residues, as the fungus has colonized the plant once the plant reaches reproductive stages (Backman et al., 1985). Chambers, as cited by Backman et al. (1985), also found that contact fungicides such as Bravo (a.i. chlorothalonil) or the systemic fungicides Quadris (a.i. azoxystrobin) and Topsin-M (a.i. thiophanate-methyl) were effective when applied during early vegetative growth stages.

Weed populations can also contribute to soybean stem canker incidence. Roy and Miller (1983) observed that cotton (*Gossypium hirsutum*) could act as an asymptomatic host to *Diaporthe* and *Phomopsis* species. This led Black et al. (1996) to investigate other potential hosts for southern soybean stem canker. It was found that stem canker can infect black nightshade (*Solanum nigrum* L.), spiny amaranth (*Amaranthus spinosus* L.), tall morning-glory (*Impomoea purpurea* L.), sicklepod (*Cassia obtusifolia* L.), small-flower morning-glory (*Jacquemonita tamnifolia* L.), Northern joint-vetch (*Aeschynomene virginica* L.), prickly sida (*Sida spinosa* L.), entire-leaf morning-glory (*Ipomoea hederacea* var. *integriuscula* Gray), pitted morning-glory (*Ipomoea lacunosa* L.), redweed (*Melocia corochorifolia* L.), hairy indigo (*Indigofera hirsuta* Harvey), and wild poinsettia (*Euphorbia cyathophora* Murray. Control of these and other weeds can reduce disease incidence by removing non-soybean reservoirs of the pathogen. Dense weed populations can also provide a shaded, humid environment that is favorable for disease development (Black et al., 1996).

Soybean Resistance to *D. aspalathi*

By far, the most effective method for management of soybean stem canker is to use resistant soybean cultivars (Freitas et al., 2002). Resistance to *D. aspalathi* in soybean is controlled by at least five major, dominant genes: *Rdm1*, *Rdm2*, *Rdm3*, *Rdm4* and *Rdm5* (Kilen and Hartwig, 1987; Bowers et al., 1993; Tyler, 1996). These resistance genes do not all perform equally against different isolates of *D. aspalathi* (Kilen et al., 1985). Furthermore, genes that provide resistance to *D. aspalathi* might not provide resistance to *D. caulivora* (northern soybean stem canker causal agent) (Kilen et al., 1985). The *Rdm* genes do not prevent infection; rather, they activate a host response restricting the growth of the fungus, allowing the plant to develop asymptomatically (Ploetz and Shokes, 1987). Differential reactions of soybean cultivars to southern soybean stem canker were first observed in the 1950s (Keeling, 1982; Weaver et al., 1984). The cultivar Tracy-M was designated as resistant as it exhibited lower levels of stem canker under field conditions. Another close relative of this cultivar, Bay, was also observed to have resistance to soybean stem canker. These cultivars are suspected to have had inherited their resistance from a shared ancestor, CNS (Keeling, 1982).

In a breeding study performed by Chiesa et al. (2012) in Argentina, *Rdm4* and *Rdm5* were found to be linked, and therefore, often inherited together. Two F_2 segregating populations were developed from soybean cultivar Hutcheson which contains both *Rdm4* and *Rdm5*. These populations each contained only either *Rdm4* or *Rdm5*. Differential reactions were observed between the populations when the two populations were tested with different *D. aspalathi* isolates; *Rdm4* segregant lines were found to be

resistant to isolate CE112 while *Rdm5* segregant populations were found to be resistant to isolate CE109. Hutcheson was found to be resistant to both isolates (Chisea et al., 2012).

Discovery of these resistance genes was initially accomplished through observation of phenotypic ratios of resistance and susceptibility to soybean stem canker in mapping populations (Kilen et al., 1985). A cross was performed between the resistant parent, Tracy-M, and the susceptible parent, J77-339. An F_2 population was generated from these plants and grown in a field free from soybean stem canker. Both parents and individuals from the F_1, F_2, and 20 of each of the 199 F_3 lines were inoculated under field conditions. The F_1 progeny exhibited the same strong resistance of their Tracy-M parent. The F_2 generation exhibited a 15:1 resistant to susceptible segregation ratio with a chi-squared value of 0.04. These ratios indicated that at least two major dominant resistance genes are present in Tracy-M (Kilen et al., 1985). These were confirmed as distinct genes and were named *Rdm1* and *Rdm2* (Kilen and Hartwig, 1987).

Another study of the inheritance of resistance to soybean stem canker led to the discovery of two distinct resistance genes, one each in the cultivars Dowling and Crockett (Bowers et al., 1993). These were used as resistant parents in crosses with susceptible parent cultivars Coker 338 and Johnston. In greenhouse evaluations of F_2 progeny from crosses of either Dowling or Crockett with the two susceptible parents, phenotypic ratios displayed was 3:1 resistant to susceptible. The $F_{2:3}$ families fit a segregation ratio a 1:2:1 (resistant, segregating, susceptible) ratio. When Crockett was crossed with Dowling, the F_2 generation's ratio was 15:1 and the F_3 generation's ratio was 7:8:1. This indicated that the resistance in each cultivar was distinct (Bowers et al., 1993). Finally, both Crockett and Dowling were crossed with Tracy-M to determine if their resistance was different

from *Rdm1* and *Rdm2*. The segregation ratios of 63:1 in the F_2 generation and of 37:26:1 in the F_3 generated from this cross confirmed that the resistance in Dowling and Crockett were distinct. *Rdm3* and *Rdm4* gene symbols were used to designate the resistance to soybean stem canker provided by Crockett and Dowling, respectively (Bowers et al., 1993).

The location of these resistance genes in the soybean genome has been investigated to better understand heritability and improve breeder selection for resistance to soybean stem canker. Shearin (2007) performed molecular mapping of several of these resistance genes. Bulk-segregant analysis was conducted using SSR markers on an F_2 population produced from susceptible by resistant crosses to locate resistance genes *Rdm1*, *Rdm3*, and an unknown *Rdm* gene (*Rdm?*). Both *Rdm3* and *Rdm?* were mapped to the top of the LG-B2 (chromosome 14) approximately 6 cM from each other. Rdm1 was mapped to the LG-D1b (chromosome 2) within 10 cM of the Satt428 marker (Shearin, 2007). In a separate study, *Rdm2* and *Rdm 4* were mapped to chromosome 2 on different regions of the chromosome (Shearin, 2007). Mapping of *Rdm5* has not been completed at this date, but due to its linkage to *Rdm4* it is likely that it is located on chromosome 2 (Chisea et al., 2013).

Greenhouse Inoculation Assay for Stem Canker

Due to the long incubation period between infection and symptom development, a quicker greenhouse was thought necessary for studying stem canker (Crall, 1952). *D. aspalathi* is a weak, opportunistic pathogen; thus, it often requires a wound for quicker penetration and proliferation (Ploetz and Shokes, 1987). A commonly used inoculation method for soybean stem canker assays is Keeling's toothpick method modified from

Crall (1952) or variations of that method (Keeling, 1982; Backes et al., 2005). Crall's method was used to inoculate cornstalks with the causal agent of charcoal rot, *Macrophomina phaseolina,* and soybean stems with the stem canker causal agent, *Diaporthe aspalathi.* Toothpicks were boiled in several changes of tap water, then pressed into agar with actively growing fungus. Colonized toothpick tips were then inserted into steel probe punctures in the stem or into stubs of cut petioles approximately 15-20 mm from the stem. Inoculated stems sealed with petrolatum produced cankers on 81% of infected plants; inoculated petiole stubs sealed with petrolatum produced cankers on 42% of infected plants; inoculated petiole stubs with no petrolatum applied had cankers on 1% of infected plants. It was concluded that this is an easily adapted, reproducible inoculation method, especially for a weak pathogen, such as *D. aspalathi*, that requires a wound as a point of entry (Crall, 1952). In Keeling's method, flat toothpicks were sterilized through boiling through three cycles of boiling water, dried, and placed in 150-ml vials. Each vial was filled with 25 ml of potato-dextrose liquid medium and autoclaved. Once the toothpicks had cooled, they were submerged in a potato dextrose liquid medium and seeded with a *D. aspalathi* isolate. Mycelium was allowed to grow over the toothpicks. Soybean plants were inoculated by first drilling a vertical hole in the stem 10 cm above the soil with a dissecting needle and the infested toothpicks were inserted into the hole. No sealing agent was used (Keeling, 1982). Ploetz and Shokes (1989) further simplified the toothpick inoculation method by autoclaving toothpicks in water and simply pressing the toothpicks onto the surface of PDA that was then seeded with fungus.

The toothpick inoculation methods have 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 J77-339. Bowers et al. (1993) observed 12% of resistant Dowling seedlings susceptible to stem canker using the toothpick assay and attributed this to mixed seed. Kilen and Hartwig (1987) reported one resistant J-77-339 seedling and 17 susceptible seedlings after stem inoculations. In contrast, Tyler (1996) observed consistent phenotype reactions of two resistant and four susceptible (including J77-339) soybeans using Keeling's toothpick inoculation procedure. Pioli et al. (2003) used the method in characterizing isolates of *Diaporthe phaseolorum* from the soybean producing area of Argentina. However, plants were only categorized as susceptible if cankers were produced or if plant death occurred. In a study of nine Iowan isolates of *D. caulivora* (northern stem canker causal agent), the isolates were assessed using four components of pathogen aggression (Lu et al. 2010). The variables considered were incubation period (time from inoculation to the time when lesion length was ≥5 mm), the rate of lesion expansion (the slope of the regression line relating growth of lesion length with respect to time after inoculation), final lesion length, and the time from inoculation to plant death (Lu et al., 2010).

Other greenhouse inoculation methods have also been utilized in studies of soybean stem canker with varying degrees of success and reproducibility (Backman et al., 1985; Weaver et al., 1988). In two studies of soybean stem canker resistance genes, Chisea et al. (2009, 2013) inoculated seedlings at the trifoliate leaf stage. They wounded the hypocotyl by making an incision in the outer layer of the stem parallel to the hypocotyl's axis. A 1.5 x 1.5 mm portion of mycelium was inserted into the incision, and

the wound was immediately sealed with petroleum jelly to avoid dehydration of the stem. Seedlings were then placed in a humidity chamber to expedite fungal infection (Chisea et al., 2013). This method is very time consuming and labor intensive. In addition, a disease phenotype was not observed on all susceptible J77-339 seedlings (Chisea et al., 2009, 2013). Thus, it would not be reliable to evaluate breeding populations for stem canker resistance.

Damicone et al. (1987) used a conidial suspension in a study of free moisture's effect of the development of northern soybean stem canker. *D. caulivora* was cultivated on potato-carrot agar acidified to pH 4.5. Ascospores and conidia were collected by flooding of plates with water and agitation of fungal material. The suspension was used to inoculate 35-day-old plants immediately after preparation. This method is highly suitable for studies of the disease and conditions affecting disease proliferation (Damicone et al., 1987). Conversely, it would not be suitable for inoculations of large number of breeding lines.

An improved greenhouse inoculation procedure for southern stem canker that is rapid and reproducible would allow for screening large numbers of plants to assist future identification of resistant germplasm and assist the long-term goals of developing high-yielding, stem canker resistant soybean varieties.

Research Objectives

The research objective are to: 1) develop an effective protocol to evaluate the ability of *D. aspalathi* to infect soybean seedlings and 2) assess virulence of *D. aspalathi* collected from the three UGA stem canker nurseries with the improved inoculation assay.

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Figure 1.1. Interveinal chlorosis characteristic of southern soybean stem canker in G81-2057 seedlings in Griffin, GA 2014.

Figure 1.2. Soybean seedlings inoculated with *D. aspalathi* CA13 using the toothpick method. Infected seedling showing elongated lesion (left). No fungus control (right).

Table 1.1. Nomenclature of the southern stem canker pathogen in publications from 1952 to present.

Author	Year	Location	Name of Fungus
Crall	1952	Not disclosed	*D. phaseolorum* var. *batatis*
Kmetz et al.	1978	MN	*D. phaseolorum* var. *caulivora*
Keeling et al.	1982	MS	*D. phaseolorum* var. *caulivora*
Roy and Miller	1983	MS	*D. phaseolorum* f. sp. *caulivora*
Backman et al.	1984	AL	*D. phaseolorum* var. *caulivora*
Phillips	1984	GA	*D. phaseolorum* var. *caulivora*
Weaver et al.	1984	AL	*D. phaseolorum* f. sp. *caulivora*
Keeling et al.	1985	OH	*D. phaseolorum* var. *caulivora*
Kilen et al.	1985	MS	*D. phaseolorum* var. *caulivora*
Ploetz and Shokes	1985	FL	*D. phaseolorum* f. sp. *caulivora*
Rothrock et al.	1985	GA	*D. phaseolorum* f. sp. *caulivora*
Russin and Boethel	1986	LA	*D. phaseolorum* f. sp. *caulivora*
Damicone et al.	1987	LA	*D. phaseolorum* var. *caulivora*
Higly	1987	IA	*D. phaseolorum* var. *caulivora*
Kilen and Hartwig	1987	MS	*D. phaseolorum* var. *caulivora*
McGee and Biddle	1987	IA	*D. phaseolorum* var. *caulivora*
Ploetz and Shokes	1987a	FL	*Diaporthe phaseolorum*
Ploetz and Shokes	1987b	FL	*D. phaseolorum* var. *caulivora*
Keeling et al.	1988	MS	*D. phaseolorum* var. *caulivora*
Rothrock et al.	1988	GA	*D. phaseolorum* f. sp. *caulivora*

Weaver et al.	1988	AL	*D. phaseolorum* f. sp. *caulivora*
Kulik et al.	1989	MD	*D. phaseolorum* var. *caulivora*
Ploetz and Shokes	1989	FL, MS	*D. phaseolorum* f. sp. *meridionalis*
Smith and Backman	1989	AL	*D. phaseolorum* f. sp. *meridionalis*
Damicone et al.	1990	LA	*D. phaseolorum* var. *caulivora*
Subbarao et al.	1992	LA	*D. phaseolorum* f. sp. *caulivora*
Bowers et al.	1993	TX	*D. phaseolorum* var. *caulivora*
Black et al.	1996	LA	*D. phaseolorum* var. *caulivora*
Fernandez and Hanlin	1996	GA	*D. phaseolorum* var. *meridionalis*
			D. phaseolorum var. *caulivora*
Tyler	1996	MS, TN, GA, AR	*D. phaseolorum* f. sp. *meridionalis*
Pioli et al.	1997	Argentina	*D. phaseolorum* var. *meridionalis*
Rupe et al.	1999	AR	*D. phaseolorum* f. sp. *meridionalis*
Pioli et al.	2003	Argentina	*D. phaseolorum* var. *meridionalis*
Si et al.	2004	WI	*D. phaseolorum* var. *meridionalis*
van Rensburg et al.	2006	South Africa	*D. aspalathi*
Kanematsu et al.	2007	Japan	*Diaporthe* sp.
Chisea et al.	2009	Argentina	*D. phaseolorum* var. *meridionalis*
			D. phaseolorum var. *caulivora*
Wrather et al.	2009	U.S.	Stem Canker
Lu et al.	2010	Iowa	*D. phaseolorum* var. *caulivora*
Santos et al.	2011	Croatia	*D. caulivora*, *D. phaseolorum*

Chisea et al.	2013	Argentina	*D. phaseolorum* var. *meridionalis*
Wrather et al.	2003-5	U.S.	Stem Canker

Chapter 2

EFFECTS OF A MODIFIED INOCULATION ASSAY ON DEVELOPMENT OF SOUTHERN STEM CANKER DISEASE ON SOYBEAN SEEDLINGS

Southern stem canker of soybean, caused by *Diaporthe aspalathi* (syn. *Diaporthe phaseolorum* f.sp. *meridionalis*), inflicted large losses to growers in the southeastern United States in the 1980s. From 1980-89, losses of approximately 854,000 metric tons in soybean yield were attributed to southern soybean stem canker (Wrather et al., 1995). In severe cases, field losses reached levels of up to 80% (Krausz and Fortnum, 1983). In 2003, a loss of $67.1 million due to southern soybean stem canker was reported across southeastern U.S. (Wrather, 2009). Combined losses of northern and southern stem canker in the U.S. amounted to 12.7 million bushels in 2014 (http://extension.cropsciences.illinois.edu/fieldcrops/diseases/yield_reduct-ions.php; verified 6 November 2016). 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).

Stem canker was originally presumed to be caused by one pathogen, but is now recognized as two distinct diseases: northern stem canker caused by *Diaporthe caulivora* (Athow and Caldwell, 1954) and southern stem canker caused by *Diaporthe aspalathi* (van Rensburg et al., 2006). The species have been distinguished and placed into different

phylogenetic clades due to sequence differences in the internal transcribed spacer (ITS) region and translation elongation factor (van Rensburg et al., 2006; Zhang et al., 1998). They also differ in morphology; *D. aspalathi* produces pycnidia and *D. caulivora* does not (Pioli et al., 2003).

Diaporthe aspalathi is an ascomycetous fungus that overwinters on soybean crop debris, infecting the following crop by rain splashing of ascospores or conidia onto wounded stems and petioles (Ploetz and Shokes, 1987a). Infection typically takes place early in the spring with symptoms appearing much later during pod filling or reproductive stages of the plant (Fehr et al., 1971). Symptoms include development of reddish brown lesions on the stem, which may lengthen along the stem and become sunken and necrotic. Leaves may develop interveinal chlorosis and can eventually become necrotic, remaining attached to the stem. The infected vascular tissue blocks the translocation of water to the seed-bearing portion of the soybean, 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 soybean stem canker when applied to cultivars of intermediate susceptibility (Weaver et al., 1984). However, the most effective way of disease management is the planting of soybean varieties with resistance to *D. aspalathi* in conjunction with the burial of inoculum by tillage (Backman et al., 1985; Tyler, 1995). This resistance is conditioned by at least five single dominant genes designated as *Rdm1*, *Rdm2*, *Rdm3*, *Rdm4*, and *Rdm5* (Chisea et al., 2009).

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 agar (Keeling, 1982) while Ploetz and Shokes (1989) utilized toothpicks colonized on agar plates. Infection of susceptible soybean using the toothpick inoculation technique has been variable with some studies observing consistent disease incidence (Tyler, 1996; Kilen et al., 1985) and others reporting conflicting phenotypes within same susceptible check (Keeling, 1985; Ploetz and Shokes, 1989**)**. For example, Keeling's method resulted in 7% of susceptible J77-339 seedlings being classified as resistant. The toothpick inoculation method produced mixed results with Bragg soybean: 39 plants were classified as resistant, while 61 were classified symptomatic or dead (Keeling, 1985). 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 inoculation methods on the ability of *D. aspalathi* to cause disease in susceptible soybean line G81-2057; and develop an effective protocol for screening of soybean breeding lines for stem canker resistance.

Materials and Methods

Diaporthe aspalathi isolates

During the fall of 2013, stems from susceptible soybean cultivar/line Hutton and G81-2057 showing stem canker symptoms were collected from UGA soybean stem canker nursery in Calhoun, Georgia. Stem sections (30-40 cm long) were collected from

symptomatic plants. Stems were then cut into ~5 cm pieces and surface sterilized (one minute in 70% ethanol, followed by two minutes in 1% sodium hypochlorite), removed and blotted dry. Individual pieces were placed onto acidified potato dextrose agar. Putative *D. aspalathi* isolates growing from the stem sections were transferred to fresh acidified PDA. Fungal isolates with the morphological characteristics of *D. aspalathi* were subsampled by hyphal tips onto acidified PDA. These isolates exhibited white to grey, tan, or white hyphal growth with and without pycnidia development (van Rensburg et al., 2006).

Identification of putative *D. aspalathi* isolates (CA10-13 and CA13-13) was done based on DNA sequence of the internal transcribed spacer region. Each isolate was cultured on PDA for 7-14 days and DNA was extracted using the Chelex® method (Walsh et al., 1991). A small amount of hyphae was added to 300 ml of 10% Chelex® solution. Each solution was vortexed, centrifuged for 15 sec, and then incubated for 20 minutes at 95^0C. Samples were vortexed again, centrifuged for 15 sec and DNA recovered from the supernatant liquid.

PCR amplification of the internal transcribed spacer (ITS) was performed using primers ITS1 (sequence 5'-3' TCCGTAGGTGAACCTGCGG) and ITS4 (sequence 5'-3' TCCTCCGCTTATTGATATGC) (Bertini et al., 1999). The samples were heated at 94^0C for two min. Forty cycles were then performed of 94^0 C for 30 sec, 55^0C for 30 sec, and 72^0C for 30 sec. Samples were then held at 72^0C for five min. PCR product was checked for amplification and correct amplicon size (500-600 bp) by 1% agarose electrophoresis gel. Amplified product was purified using ExoSAPit reagent (Affymetrix; Santa Clara,

CA) and sequenced at Georgia Genomics Facility using an applied Biosystems 3730xl 96-capillary DNA Analyzer (Thermo Fisher Scientific; Waltham, MA).

The type isolate of *D. aspalathi* (isolate DPM 1F; ID number ATCC200236) originally isolated in Georgia (Fernandez and Hanlin, 1996) was acquired from the American Type Culture Collection and included in the validation experiment. Long-term storage of isolates was done by lyophilizing *D. aspalathi*-infected stem tissues. Short-term storage was on PDA slants at 4^0C at UGA plant pathology facilities in Griffin, GA. Fresh, working inoculum was maintained by subsampling isolates onto new PDA every two to three weeks.

Plant materials

The southern stem canker susceptible soybean line G81-2057 was utilized in the inoculation assay tests, as it has proved highly susceptible in previous experiments and stem canker nurseries (Backman et al., 1985). Seeds were planted in Jolly Gardener Pro-Line C/B growing mix (Atlanta, GA) in 10-cm x 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 to prevent plant crowding and r simplify disease rating (Harris et al., 2014). Three seeds were planted in each pot and seedlings were thinned to two seedlings per pot at 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 when the seedlings reached to growth stage V2 during this period unless otherwise specified (Keeling, 1982). 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 μg mL^{-1} N from a stock of 20-20-20 Scotts Peters water soluble fertilizer.

Optimization of inoculation assay

Soybean seedlings were inoculated with *D. aspalathi* by inserting fungus-colonized toothpicks into the stem of susceptible G81-2057 soybean seedlings. Toothpick colonization described by Keeling (1982) and a modified version (Ploetz and Shokes, 1989) were included for comparison in the experiment testing the effect growth media recipe, agar use, and wound sealant use to observe possible improvements. Briefly, Keeling (1982) boiled toothpicks in water three times, and then autoclaved the toothpicks in potato dextrose liquid medium. Fungal inoculum was added to the liquid medium and allowed to colonize the toothpicks for 15 days at 21^0 C. Ploetz and Shokes (1989) autoclaved toothpicks in deionized water and then placed the toothpicks on the surface of PDA that was then seeded with plugs of *D. aspalathi* mycelium from PDA slants and allowed to grow for two weeks at 20-25^0C.

Plant growth stages at time of inoculation were tested to determine if more consistent or expedient disease development could be achieved than that inoculated at growth stage V2 used by Keeling (1982). Different stem locations of inoculation were tested to compare to the inoculation below the cotyledon utilized by Keeling (1982). Toothpicks were soaked and autoclaved in water prior to inoculation in Ploetz and Shokes (1989), but improved inoculum colonization of toothpicks could be potentially achieved by soaking them in liquid growth media (Table 2.1). Initial experiments were conducted using toothpicks colonized by *D. aspalathi* CA13-13 on PDA for two weeks

by following the protocol reported by Ploetz and Shokes (1989). Except where noted, three-week old plants were inoculated by inserting toothpicks into the stem above the cotyledon.

To optimize the inoculation assays, five experiments were conducted. The first experiment tested the effect of using petroleum jelly on the toothpick inoculation sites followed by either placing the plants in a 72 h incubation in a high-humidity chamber (plastic enclosure 3.0 m x 1.0 m x 0.8 m) or no humidity chamber treatment on subsequent disease development. Ploetz and Shokes (1989) did not use either of these treatments; only wound sealant was utilized by Keeling (1982). The bottoms of the chambers were flooded with water and the chambers were sealed. Average temperature in the chambers was 21^0 C and average relative humidity 95%.

The second experiment compared the effect of plant age on disease development by inoculating two, three, and four-week-old soybean seedlings (V2, V3 and V4 growth stages). Wound sealant (petroleum jelly) was used and inoculated plants were placed into a humidity chamber for 72 h then placed on a greenhouse bench. This experiment tested the efficiency of inoculation at growth stage V2 that was utilized by Keeling (1982).

A third experiment tested the effect of inoculation location on the seedlings by inserting *D. aspalathi*-colonized toothpicks into the stem above or below the cotyledon, into the leaf axis, both above and below the cotyledon (two inoculation points/plant), above the cotyledon and into the leaf axis, and below the cotyledon and into the leaf axis. Plants were put in a humidity chamber for 72 h and then placed on a greenhouse bench.

A fourth experiment was conducted to compare the toothpick colonization procedures of Keeling (1982) and Ploetz and Shokes (1989) to toothpicks colonized by

D. aspalathi in liquid or on agar-amended malt dextrose medium, potato dextrose medium, or soybean stem lima bean medium (Phillips and Boerma, 1981) on subsequent production of stem canker symptoms on soybean seedlings. The effect of wound sealant was also assessed with each of these media recipes. Toothpicks were autoclaved in distilled water three times and then either placed on the surface of agar medium or submerged in 100 ml liquid medium. Agar from the edge of two-week old culture of *D. aspalathi* CA13-13 on PDA was then added to the surface the liquid media or center of agar plates. Seeded media was incubated for two to three weeks at room temperature. The *D. aspalathi*-colonized toothpicks were then inserted into the stem, above the cotyledon, of three-week-old soybean seedlings. Each media treatment was then sealed with petroleum jelly or left untreated and placed in a humidity chamber for 72 h to expedite fungal growth. Plants were moved to a greenhouse bench. These four experiments were scored for disease incidence at two and three-weeks after inoculation.

A fifth experiment compared three isolates of *D. aspalathi* (CA10-13, CA13-13, and DPM 1F) cultured on toothpicks autoclaved in oxgall liquid medium (Difco, Sparks, MD) and then placed on oxgall agar, toothpicks autoclaved in soybean stem lima bean liquid medium and then placed on soybean stem lima bean agar, or toothpicks autoclaved in potato dextrose liquid medium and then placed on PDA. These treatments were compared to toothpicks autoclaved in water (Keeling, 1982) and then placed onto the surface of three agar media (oxgall, soybean stem lima bean, or potato dextrose). The colonized toothpicks were inserted into the stem, below the cotyledon of 3-week-old soybean seedlings. Toothpick point of entry wounds were sealed with petroleum jelly and

plants were placed in high humidity chambers for 72 h. Plants were moved to a greenhouse bench and disease was two, three and four weeks post-inoculation.

Validation of the developed inoculation assays

To validate the protocol developed in previous experiments, a panel of 24 soybean lines was selected to assess the reliability and reproducibility of the assay using three *D. aspalathi* isolates (CA10-13, CA13-13, and DPM 1F). Resistant soybean lines included in the validation study were Tracy-M (*Rdm1*/*Rdm2*), Crockett (*Rdm3*), Dowling (*Rdm4*), and Hutcheson (*Rdm4/Rdm5*), PI 398469 (*Rdm?*) and Benning (*Rdm?)* (Tyler, 1996, Shearin, 2007, Boerma et al., 1997). Southern stem canker susceptible lines/cultivars included were Bedford, Bragg, Braxton, Centennial, Coker 237, Coker 338, Davis, Essex, G81-2057, Hartwig, Hutton, J-77-339, Kirby, Santa Rosa, and Woodruff (Backman et al., 1985, Keeling et al., 1985). PI 230976 and G12PR-214 were included with no previous records of resistance or susceptibility to southern soybean stem canker. *D. aspalathi* was first cultured on oxgall media. Toothpicks were soaked and autoclaved in oxgall liquid medium then placed on oxgall media amended with agar and incubated at room temperature for two to three weeks. Three-week-old seedlings were inoculated above the cotyledon with colonized toothpicks and petroleum jelly was used to seal the entry point. Plants were then placed in a high humidity chamber for 72 h to expedite fungal growth. Disease incidence was scored at two, three, and four weeks post-inoculation, respectively.

Experimental design

The first two experiments (humidity and sealant use, soybean growth stage at time of inoculation) included two replicates of 24 seedlings (12 pots with two seedlings per

pot) for each treatment. Each flat contained only one treatment due to the nature of these treatments and the difficulty of separation of treatment levels. Experiment 1 included four unique treatments (humidity chamber + wound sealant, wound sealant only, humidity chamber only, neither) with a negative control. Experiment 2 comprised of the treatment with three growth stages, with growth stage V2 treated as the standard for comparison. Complete randomized block design was used. Flat assignment for each treatment was randomized within each replication with a total of 2 replicates.

Experiment 3 consisted of six inoculation location treatments with six replicates per treatment. The treatments were arranged with a complete randomized block design. Each treatment had eight seedlings in each replicate and was randomly assigned a position in a flat within each replicate. In experiments 2 and 3, certain treatments caused significant amounts of lost data (stem snapping resulting in seedling death), and the damage was assessed and analyzed.

Both experiments 4 and 5 utilized a complete randomized block design. Experiment 4 included three treatment factors: inoculum preparation media recipe (soybean stem lima bean, potato dextrose, malt dextrose), agar use in inoculum preparation media, and wound sealant use. These factors were combined for a total of unique 12 treatment combinations plus a negative control of an uninfected toothpick. Each treatment combination was replicated six times, with eight seedlings per replicate and treatment. Treatments were randomly assigned flat positions within each replicate. For experiment 5, there were also three treatment factors. The effects of toothpick treatment (water or liquid medium soaked), *D. aspalathi* isolate (CA10-13, CA13-13, DPM 1F), and media recipe (oxgall, soybean stem lima bean, potato dextrose) on stem

canker incidence were assessed in conjunction with one another. This resulted in 18 unique treatment combinations. Each treatment combination was replicated four times, with one replicate consisting of 12 seedlings (six pots with two seedlings per pot). Treatments were randomly assigned flat positions within each replicate.

A split plot experimental design was employed in the validation experiment. Three *D. aspalathi* isolates were used as the whole-plot factor, which were replicated twice. Soybean lines were treated as a split-plot factor. Each of the 24 soybean lines was replicated twice within each of the whole plots. Soybean lines were randomized within the whole plot of *D. aspalathi* isolate and each soybean line replicate consisted of 12 seedlings. The most effective treatments developed from previous five experiments were used for this validation study. Specifically, the inoculum was prepared on oxgall agar medium and toothpicks were soaked and autoclaved in oxgall liquid medium prior to plating. Seedlings were inoculated at growth stage V2 between the cotyledon and first trifoliate. Inoculation wounds were sealed with petroleum jelly and plants were placed in humidity chambers for 72 h after inoculation. Plants were then moved to greenhouse benches and disease incidence was assessed at two, three, and four weeks after inoculation.

All above five experiments and validation study were conducted with two planting dates in our Griffin greenhouse facility and similar greenhouse condition was employed for these experiments.

Data collection and analysis

Soybean stem canker symptoms were rated on a binomial scale with a score of 1 for symptomatic plants and a score of 0 for non-symptomatic plants. Symptomatic plants

were defined as having a lesion extending at least 10 mm from the inoculation site and/or plants displaying the characteristic interveinal chlorosis of southern soybean stem canker (Fig 2.2). This scale was used due to high variability of lesion length within treatments.

Analysis was done using SAS Version 9.3 Proc GLM, assessing the percentage of disease incidence per replicate within each treatment for each experiment individually (SAS Institute Inc., Cary, NC). Treatments causing significant effects (P>0.05) on disease incidence percentages were further analyzed to determine which treatment level caused significantly higher disease incidence. In experiments utilizing multiple treatments, interactions between treatments were also evaluated. Plants that snapped and rendered un-ratable were treated as missing data.

Results

Optimization of Inoculation assay

In each of the five experiments, planting dates were of no significant effect (*P*>0.05). The effect of a petroleum jelly wound sealant at the inoculation site above the cotyledon in conjunction with a 72-h incubation in a high humidity chamber resulted in significantly more diseased seedlings than use of the humidity chamber alone and use of neither humidity chamber nor wound sealant by three-weeks post-inoculation (Table 2.2). Use of wound sealant only and use of sealant + humidity chamber did not significantly differ from each other, but using both resulted in objectively highest disease incidence (Table 2.2). It was determined that both the wound sealant treatment and humidity chamber treatments would be utilized in further studies.

The seedling growth stage at time of inoculation was observed to significantly effect disease at two and three weeks post-inoculation (Table 2.3). Plants inoculated at

V1 growth stage exhibited 42.4% and 73.9% disease incidence at two and three-weeks post inoculation respectively. Seedlings inoculated at V2 growth stage had 95.1% and 100% disease incidence at two and three-weeks post-inoculation, respectively. Disease symptoms took longer to develop in plants inoculated at V3 growth stage, with 27.0% of plants symptomatic at two weeks post-inoculation. Significant differences were observed in damage caused to seedlings at the point of inoculation (Table 2.3). Inoculation of plants at V1 growth stage resulted in significantly more snapped stems than older seedlings. For this reason, inoculation of seedlings at growth stage V2 was deemed most appropriate for expedient and consistent development of disease and low amounts of stem snapping.

The inoculation site on the seedling stems had a significant effect on disease development and physical damage to plants (Table 2.4). A single inoculation above or below the cotyledon resulted in 100% diseased seedlings at three-weeks post-inoculation and lowest amount of snapped seedlings. It should be noted that these seedlings were inoculated at growth stage V2, inoculation site was sealed with petroleum jelly, and plants were placed in humidity chambers for 72 h post inoculation for all location treatments. Inoculum was prepared on potato dextrose media amended with agar. Inoculation at the top leaf axis caused significantly lower seedling disease at two and three-weeks post-inoculation than all other treatments. The highest amount of damaged seedlings was also observed with the top leaf axis (43.7%). Seedling losses to mechanical damage were significantly higher for all combination treatments compared to the single inoculations above or below the cotyledon. Inoculation above the cotyledon was selected

for further utilization due to its high disease incidence, relative low stem snapping, and ease of application.

Significant treatment effects ($P<0.05$) were observed with the presence or absence of agar, the use of wound sealant, and the interaction between agar × sealant uses on the development of stem canker disease on seedlings based on ANOVA (Table 2.5). By three-weeks post-inoculation, no differences were observed between culturing *D. aspalathi* on toothpicks with potato dextrose, malt dextrose, or lima bean soybean stem growth media ($P=0.22$) (Table 2.5). However, a significant improvement in disease development was observed across these growth media with media solidification with agar. Using petroleum jelly as a sealant improved disease incidence significantly compared to not sealing inoculation sites (89.9% compared to 51.6% disease incidence at three weeks post inoculation). Use of agar in inoculum preparation media significantly improved disease incidence ($P<0.05$) at both two and three weeks after inoculation (Table 2.6).

Based on the results from the previous four experiments, three treatment factors were combined to make 18 unique treatment combinations in the fifth experiment. The treatments included *D. aspalathi* isolate (CA10-13, CA13-13, and DPM 1F), pre-soaking treatment of toothpicks (soak in liquid growth media versus water), and growth medium (oxgall, soybean stem lima bean, and potato dextrose). Inoculation was performed at seedling growth stage V2 above the cotyledon and below the first trifoliate. Wound sealant was applied to the inoculation site and plants were place in humidity chambers for 72 h post-inoculation. It was theorized that soaking of toothpicks in liquid growth media could allow for fungus to colonize the inside of the toothpick as well as the outside,

making for a higher inoculum load. Multiple liquid medium recipes (potato dextrose, oxgall, soybean stem lima bean) were tested to observe potential improvements of suitability for colonization. A significant increase in disease incidence ($P<0.05$) was observed at four weeks post-inoculation for both growth medium and pre-soaking toothpicks (Table 2.7). At four weeks post-inoculation, pre-soaking in liquid growth media resulted in significantly higher disease incidence (97.2% vs. 88.7%) across other treatment factors than soaking toothpicks in water (Table 2.8). Inoculum prepared on oxgall media and soybean stem lima bean media caused significantly higher levels of disease than inoculum prepared on potato dextrose media by four weeks post inoculation (Table 2.8) across other treatment factors as well. Oxgall media was selected for use in further studies due to the difficulty of soybean stem lima bean media preparation. Soaking and autoclaving toothpicks in liquid medium as opposed to water was selected as a treatment for use in further experiments due to its significant increase in disease incidence. There was no difference observed between pathogen isolate at three and four-weeks post-inoculation (data not shown).

Validation of the developed inoculation assay

A combination of treatments in this study that increased the percentage of disease seedlings was selected and tested on a panel of six resistant and 18 susceptible soybean lines using three isolates of *D. aspalathi* (Tables 2.9 and 2.10). Specifically, inoculum was prepared on oxgall agar and toothpicks were soaked and autoclaved in oxgall liquid medium prior to plating. Inoculation was performed at growth stage V2 between the cotyledon and the first trifoliate. Inoculation sites were sealed with petroleum jelly and seedlings were placed in humidity chambers for 72 h post inoculation. Stem canker

disease was not observed on the six resistant soybean lines as expected (Table 2.10). However, significant differences in the percentage of diseased plants were observed among susceptible soybean lines at three and four weeks post-inoculation (Table 2.10). Disease progressed the slowest on soybean cultivar Braxton with significantly lower percent disease observed from 3 to 4 weeks post-inoculation. All seedlings of Bragg, Woodruff, Kirby, and Santa Rosa had stem canker disease four weeks post-inoculation. By four weeks post-inoculation there was no significant difference between diseases caused by the three isolates (CA13-13: 99.1%; CA10-13: 99.0%, and DPM 1F: 98.7%) averaged across all susceptible lines (data not shown). The high levels of disease in susceptible soybean lines across all used *D. aspalathi* isolates indicates high suitability of this inoculation method to be used to determine greenhouse susceptibility or resistance of soybean lines to various *D. aspalathi* isolates.

Discussion

Several variables in the toothpick inoculation assay of Keeling (1982) were identified as points for potential disease incidence improvement. Use of wound sealant was utilized in Keeling's inoculation method (1982) but not in Ploetz and Shokes's method (1989). The use of humidity chambers post inoculation was not included in either of these methods, but the shaded humid conditions in such chambers could expedite fungal growth. Both Keeling and Ploetz and Shokes used potato dextrose media for inoculum preparation. But it was theorized that other media recipes could result in a more vigorously growing inoculum and subsequently more consistent rates of stem canker incidence. Agar use in inoculum preparation media state has been included (Ploetz and Shokes, 1989) and excluded (Keeling, 1982) in previous experiments. Using agar in

inoculum preparation media could ensure a more even coverage of fungus across toothpicks. However, exclusion of agar (liquid growth media) could make for less labor-intensive preparation of inoculum.

Disease resistance is one of important goals in soybean breeding programs. Greenhouse screening for disease resistance is one of important procedures in a breeding workflow. Procedures should be reproducible and efficient so as to be used in a high throughput manner. *D. aspalathi* is a pathogen that requires a point of entry such as a stem wound or a leaf scar, making the toothpick inoculation method an ideal delivery system (Ploetz and Shokes, 1989). Previously used inoculation methods for southern soybean stem canker have had inconsistent results in producing disease incidence in some susceptible soybean varieties (Keeling, 1985, Ploetz and Shokes, 1989, Pioli et al., 2003**)**. Susceptible plants that do not exhibit a disease phenotype (i.e. look resistant) could affect accuracy of line selections and genetic studies for stem canker resistance. Optimized inoculation assay in this study that includes a combination of pre-soaking in liquid medium (Keeling, 1982), colonization of toothpicks by *D. aspalathi* on agar (Ploetz and Shokes, 1989), inoculating three week old seedlings above the cotyledon, sealing wounds with petroleum jelly and using a 72-h high humidity incubation to facilitate infection resulted in a high disease incidence (>98.7%) on susceptible soybean seedlings of G81-2057.

The use of a humidity chamber versus a wound sealant was tested to determine if both procedures were necessary to achieve high disease incidence in susceptible lines. Applying wound sealant is time-consuming and messy. Moving seedlings in and out of a humidity chamber contributes to mechanical damage and seedling loss. High relative

humidity treatment expedites the infection process of *D. aspalathi* (Damicone et al., 1987), while the use of petroleum jelly as a wound sealant prevents the inoculation site from potentially drying out. The percentage of diseased seedling did not differ between use of either a humidity chamber or a wound sealant or both together, and both treatments produced significantly more disease than no sealant or humidity chamber treatment. A continuous wetting period in the greenhouse increases disease incidence with *D. aspalathi* (Damicone et al., 1987, Ploetz and Shokes 1987). Damicone et al. (1987) did not observe disease on seedlings when no wetting period was provided. These particular treatments mimic the effects of wetting periods by increasing humidity (chamber treatment) and preventing dry out of inoculum and stem wound (wound sealant).

Plant age at time of inoculation significantly affected development of stem canker symptoms. Plants inoculated at growth stage V1 tended to develop disease quickly, however a significant number of plants were lost due to stem snapping which would require planting and inoculation of many extra seedlings to compensate for losses. Plants inoculated at growth stage V3 withstood mechanical damage due to the inoculation process, but disease development was far lower and delayed than that of plants inoculated at growth stage V2. Since these screenings would ideally be completed in the shortest amount of time possible with the lowest amount of lost data possible, growth stage V2 proved an ideal time for stem canker inoculation. Keeling (1982) inoculated seedlings at 10 days after planting (~V2). This resulted in 7% mis-phenotyped J77-339 and 39% mis-phenotyped Bragg. This difference may have been due to the liquid medium used by Keeling, while agar amended media was used in this trial. Our results demonstrated

highest disease percentage by inoculating between growth stages V2 and V3, which is consistent with the report by Ploetz and Shokes (1989).

A field study conducted by Rupe et al. (1999) tested plant age at time of inoculation as well. It was observed that disease incidence occurred with the shortest amount of incubation time when plants were inoculated at growth stage R2 with an incubation time between 34 and 41 days. Longest pathogen incubation time of 50 to 55 days occurred when plants were inoculated at growth stage V1. In all cases, symptoms did not appear until after flowering (R2). Symptom development was delayed until growth stage R5 when plants were inoculated at growth stage R2. These longer pathogen incubation times were likely due to the less aggressive spore suspension and spray inoculation technique utilized in these experiments. Conversely, highest and most severe disease incidence was observed when plants were inoculated at growth stage V6 (Rupe et al., 1999). Smith and Backman (1989) found that highest incidence occurred when artificial inoculum was applied to susceptible soybean cultivar Kirby at growth stage V3. They also observed that symptoms did not develop until reproductive growth stages were reached. Our results were also consistent with those achieved by Smith and Backman (1989) in field studies, however, the incubation time for *D. aspalathi* was far shorter in a greenhouse condition. In some cases, disease occurred in as short as two weeks in the greenhouse setting. This could be due to the fact that plants were placed in a high humidity following inoculation or that the toothpick inoculation method is more aggressive than the application of ascospores.

Location of inoculation on the seedling significantly affected disease development as well as the structural integrity of the plant stem. Previous greenhouse studies utilizing

the toothpick method typically inoculated below the cotyledon (Keeling, 1982; Pioli et al., 2003) or above the cotyledon (Ploetz and Shokes, 1987b). In the current study, a significant number of seedlings were lost due to stem snapping or toothpicks falling out of the plant in inoculation treatments of the top leaf axis. Multiple inoculations are used in greenhouse assays for soybean rust caused by *Phakopsora pachyrhizi* (Harris et al., 2015; King et al., 2016) to ensure all plants are exposed to inoculum. It was theorized that multiple inoculations at different stem sites could also increase disease development with stem canker. However, in the present study, use of multiple inoculation sites with *D. aspalathi* did not increase disease incidence significantly and resulted in losses due to mechanical damage.

The highest disease incidence was obtained using a single inoculation above the cotyledon or below the cotyledon. Ploetz and Shokes (1987) tested various inoculation locations using a spore suspension for inoculum. They observed that petiole base and stem inoculation yielded higher disease incidence than inoculation of leaves or petioles. However, there was only an infection rate of 50.5% at petiole bases (Ploetz and Shokes, 1987). This is likely due to the spore suspension spray inoculation method used, which lacks the wounding step of the toothpick method. There were also likely variable amounts of inoculum applied using this method, as spore suspension concentration was not specified (Ploetz and Shokes, 1987).

Significantly higher disease incidence was achieved on seedlings when toothpicks were colonized by *D. aspalathi* on agar medium as opposed to liquid medium. Ploetz and Shokes (1989) also observed high disease incidence with toothpicks colonized by *D. aspalathi* on agar. Toothpick colonization in liquid media (Keeling, 1982) resulted in

lower numbers of disease seedlings. Use of wound sealant was also tested in conjunction with media recipe and type so that any interaction between sealant and media could be observed. Wound sealant improved disease incidence across all treatments presumably by keeping the inoculation site from drying out.

A toothpick pre-soaking treatment was tested with the hypothesis that fungus could potentially colonize the inside of the toothpick better if it was soaked in nutrients. There were significant improvements when pre-soaking toothpicks in media as opposed to water across all media recipes and *D. aspalathi* isolates. Inoculum prepared with oxgall and soybean stem lima bean medium produced significantly higher incidence of disease than that prepared with potato dextrose media. Oxgall media was selected for the following experiment due to ease of preparation compared to soybean stem lima bean media and relatively high disease levels caused by inoculum prepared with it.

The modified toothpick assay including pre-soaking in liquid media (Keeling, 1982) and use of agar medium for toothpick colonization by *D. aspalathi* (Ploetz and Shokes, 1989) was tested on a panel of soybean lines with known resistance and susceptibility to stem canker. Seedlings were inoculated at growth stage V2 between the cotyledon and first trifoliate. Both wound sealant and 72-h humidity chamber treatments were used. Soybean lines possessing the major *Rdm* genes developed no symptoms when inoculated (Table 2.10). Differences were observed between field and greenhouse stem canker resistance reactions in the cultivars Braxton and Centennial. Braxton and Centennial were classified as resistant in field evaluations performed by Tyler (1996), however, there was lesion development reported in that study. In the current study, Braxton and Centennial were observed to have a susceptible disease phenotype with three

isolates of *D. aspalathi*. This may be indicative of some moderate field resistance that is compromised by the aggressiveness of the toothpick inoculation method. Differences may also be caused by different isolates involved in infection. The field and greenhouse reaction discrepancy of Centennial was observed and addressed by Weaver et al. (1988) who theorized that Centennial might possess genes that react differently than the major *Rdm* genes to artificial inoculation.

This optimized assay will be highly useful in future screenings of soybean lines for resistance to southern soybean stem canker. It could also be used in future virulence assessments and characterizations of *D. aspalathi* isolates. Future studies and evaluation of southern stem canker resistance could potentially include lesion length and growth over time as an indicator of isolate virulence or soybean susceptibility. The results of these greenhouse screens should also be confirmed in field assays due to the variable nature of some stem canker resistance such as the resistance observed in Centennial in the field that was compromised in the greenhouse setting (Weaver et al., 1988).

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Fig. 2.1. Toothpicks on potato dextrose agar colonized by *Diaporthe aspalathi* CA13-13 for two weeks. Inoculation preparation similar to Ploetz and Shokes (1989).

Fig. 2.2. Stem inoculation of G81-2057 soybean seedlings in greenhouse showing lesion development along stem by *Diaporthe aspalathi* CA13-13 (left) and no-fungus control (right).

Table 2.1. The variables tested in each of the five inoculation method experiments

Experiment ID	Variables Tested
1	Humidity chamber and wound sealant
2	Plant growth stage at time of inoculation
3	Inoculation location
4	Media recipe, agar, and wound sealant
5	Media recipe, toothpick soaking, *D. aspalathi* isolate

Table 2.2. The effect of the use of petroleum jelly wound sealant and post-inoculation incubation in a humidity chamber on development of stem canker disease. Seedlings of

G81-2057 were inoculated at growth stage V3 between the cotyledon and the first trifoliate. Inoculum was prepared on potato dextrose agar.

Treatment	2-Weeks Post Inoculation		3-Weeks Post Inoculation	
	Disease % (Std. Dev.)			
Wound sealant + humidity chamber	87.5 (11.1)	a	97.9 (5.1)	a
Wound sealant	75.0 (17.6)	ab	87.5 (11.1)	ab
Humidity chamber	70.8 (23.2)	ab	83.3 (18.8)	b
No Treatment	62.5 (15.8)	b	62.5 (15.8)	c
No fungus Control	0.0 (0.0)	c	0.0 (0.0)	c

*Data represent the average of 2 replicates with 24 plants per treatment/or combination in each replicate (48 plants total).

Means with the different letters are significantly different based on LSD (0.05).

Table 2.3. The effect of plant growth stages at the time of inoculation on disease incidence and mechanical damage of seedlings. Petroleum jelly was used as wound sealant, humidity chamber was used, and inoculation site was between the cotyledon and the first trifoliate leaf. Inoculum was prepared on potato dextrose agar. Growth stage V2 was treated as the standard for comparison.

	2-Weeks Post Inoculation				3-Weeks Post Inoculation			
Inoculation Growth Stage*	Disease (%)** (Std. Dev.)		Snapped Stems (%)*		Disease (%)** (Std. Dev.)		Snapped Stems (%)*	
V1	42.4 (39.7)	b	31.2	a	73.9 (22.4)	b	37.5	a
V2	95.1 (7.6)	a	0.4	b	100.0 (0.0)	a	0.4	b
V3	27.0 (12.2)	b	0.0	b	81.2 (10.4)	b	0.2	b

*Plant growth stage corresponds to one (V2), two (V3) and three (V4)

**Data represent the average of 2 replicates with 24 plants per treatment in each replicate (48 plants total).

Means with the different letters are significantly different based on LSD (0.05)

Table 2.4. The effects of inoculation position on stem canker disease incidence on stem snapping. Petroleum jelly was used as wound sealant, humidity chamber was used, and inoculation took place at soybean growth stage V3. Inoculum was prepared on potato dextrose agar. Inoculation above the cotyledon was treated as the standard for comparison.

	2-Weeks Post Inoculation		3-Weeks Post Inoculation	
Inoculation Location	Disease (%)** (Std. Dev.)	Snapped Stems (%)*	Disease (%)** (Std. Dev.)	Snapped Stems (%)*
Above Cotyledon	91.3 (10.3)	2.0	100.0 (0.0)	6.2
Below cotyledon	64.2 (16.1)	6.2	100.0 (0.0)	6.2
Top leaf axis	54.7 (16.0)	27.0	70.5 (3.2)	43.7
Above and below cotyledon	84.5 (12.9)	6.2	90.2 (6.2)	22.9
Above cotyledon and top leaf axis	82.6 (23.1)	8.3	91.6 (20.4)	14.5
Below cotyledon and top leaf axis	71.3 (19.6)	18.7	95.8 (10.2)	27.0
LSD (0.05)	21.0	13.9	19.9	23.7

*Data represent the average of 6 replicates with 8 plants per treatment/or combination in each replicate (48 plants total).

Means with the different letters are significantly different based on LSD (0.05)

Table 2.5. Analysis of variance across growth medium, agar use, and sealant use at two and three weeks post inoculation.

Week	Source	DF	Mean Square	F Value	Pr > F
2	Replicate	2	0.000	1.61	0.20
	Growth medium	2	0.011	0.33	0.71
	Agar use	1	0.728	20.98	<.0001
	Sealant	1	5.015	144.36	<.0001
	Medium*Agar use	2	0.124	3.59	0.03
	Medium*Sealant	2	0.001	0.05	0.94
	Agar use*Sealant	1	0.152	4.38	0.04
3	Replicate	2	0.107	0.12	0.8863
	Growth medium	2	0.048	1.49	0.2332
	Agar use	1	0.770	23.73	<.0001
	Sealant	1	2.599	80.07	<.0001
	Medium*Agar use	2	0.054	1.68	0.193
	Medium*Sealant	2	0.013	0.43	0.657
	Agar use*Sealant	1	0.187	5.77	0.017

Table 2.6. The effects of growth medium, presence of agar in growth medium and use of wound sealant on development of southern soybean stem canker disease. Plants were inoculated at growth stage V3 between the cotyledon and below the first trifoliate. Plants were placed in humidity chambers for 72 h post inoculation. Negative controls in which clean toothpicks were used yielded no symptoms (data not shown). There was no significant difference between growth medium recipes (data not shown).

		2-Weeks Post Inoculation		3-Weeks Post Inoculation	
Treatment		Disease % (Std. Dev.)			
Growth medium	Potato Dextrose	54.7 (34.9)	a	67.7 (30.3)	a
	Malt Dextrose	56.7 (32.7)	a	76.0 (22.9)	a
	Soybean stem lima bean	52.3 (36.9)	a	68.6 (32.7)	a
Agar in medium	No	44.4 (36.4)	b	60.4 (20.1)	b
	Yes	64.4 (29.5)	a	81.2 (20.1)	a
Use of petroleum jelly	No	27.9 (23.0)	b	51.6 (27.9)	b
	Yes	81.2 (20.5)	a	89.9 (12.2)	a

*Data represent the average of 6 replicates with 8 plants per treatment/or combination in each replicate (48 plants total). Treatment factors were individually analyzed across all others.

Means with the different letters are significantly different based on LSD (0.05)

Table 2.7 Analysis of variance across growth medium, toothpick treatment, and *D. aspalathi* isolate for three and four weeks post inoculation.

Week	Source	DF	Mean Square	F Value	Pr > F
3	Replicate	1	0.026	0.62	0.05
	Growth medium	2	0.018	2.22	0.11
	Toothpick soak	1	0.023	3.06	0.06
	Isolate	2	0.011	1.87	0.21
	Toothpick soak*Isolate	2	0.012	2.05	0.17
	Medium*Isolate	4	0.012	1.96	0.15
	Medium*Toothpick soak	2	0.005	0.57	0.47
4	Replicate	1	0.012	0.52	0.43
	Growth medium	2	0.091	4.76	0.01
	Toothpick soak	1	0.161	9.25	0.00
	Isolate	2	0.001	0.06	0.93
	Toothpick soak*Isolate	2	0.011	0.41	0.57
	Medium*Isolate	4	0.006	0.20	0.85
	Medium*Toothpick soak	2	0.036	1.65	0.18

Table 2.8. The effect of growth medium, pre-soaking toothpicks in growth medium and *D. aspalathi* isolate on development of southern stem canker disease. Inoculation took place at plant growth stage V3 between the cotyledon and the first trifoliate. Growth medium indicates the liquid medium in which toothpicks were soaked and agar they were then plated on in cases of liquid soaking treatment. Toothpick pre-soaking indicates whether the toothpicks were soaked and autoclaved in liquid media or water. There was no significant difference between *D. aspalathi* isolates.

		3-Weeks Post Inoculation		4-Weeks Post Inoculation	
Treatment		Disease % (Std. Dev.)			
Growth medium	Potato Dextrose	87.8 (11.3)	a	86.9 (23.2)	b
	Oxgall	92.3 (6.6)	a	96.5 (4.8)	a
	Soybean stem lima bean	91.9 (7.9)	a	95.4 (7.3)	a
Toothpick pre-soaking	Water	88.7 (9.9)	a	88.7 (19.7)	a
	Liquid medium	92.4 (7.8)	a	97.2 (5.6)	b

*Data represent the average of 6 replicates with 8 plants per treatment/or combination in each replicate (48 plants total).

Means with the different letters are significantly different based on LSD (0.05)

Table 2.9. Analysis of variance across growth medium, toothpick treatment, and *D. aspalathi* isolate for three and four weeks post inoculation.

Week	Source	DF	Mean Square	F Value	Pr > F
3	Replicate	3	0.055	1.65	0.177
	Line	24	2.008	4.75	<0.0001
	Isolate	2	0.107	171.63	0.009
	Isolate*Line	48	0.0100	0.86	0.717
4	Replicate	3	0.002	0.62	0.59
	Line	24	2.227	549.43	<0.0001
	Isolate	2	0.009	2.15	0.11
	Isolate*Line	48	0.289	0.00	0.02

Table 2.10. The disease incidence averaged across three *D. aspalathi* isolates for 24 soybean lines. Plants were inoculated at growth stage V3 between the cotyledon and the first trifoliate. Wounds sealant was applied to the inoculation site and plants were placed in humidity chambers for 72 h post inoculation. Inoculum was prepared on oxgall agar and toothpicks were soaked in oxgall liquid medium prior to plating.

		3-Weeks Post Inoculation	4-Weeks Post Inoculation
Soybean Line	Resistant/ Susceptible	Disease % (Std. Dev.)	
Bedford	S	89.5 (18.8)	96.5 (5.5)
Bragg	S	96.5 (6.6)	100.0 (0.0)
Braxton	MS	70.1 (22.8)	84.7 (27.0)
Centennial	MS	86.1 (18.9)	96.5 (4.2)
Coker237	S	91.6 (14.2)	98.6 (3.2)
Coker338	S	93.7 (10.7)	97.2 (6.4)
Davis	S	91.6 (14.6)	98.6 (4.8)
Essex	S	95.1 (6.6)	97.9 (3.7)
G12PR-214	S	93.0 (14.5)	99.3 (2.4)
G13-6299	S	93.7 (9.4)	97.2 (4.1)
G81-2057	S	95.1 (6.8)	98.6 (5.2)
Hartwig	S	88.8 (8.2)	92.3 (9.0)
Hutton	S	95.1 (7.5)	98.6 (4.8)
J-77-339	S	98.6 (3.2)	99.3 (2.4)
Kirby	S	92.3 (12.5)	100.0 (0.0)

Santa Rosa	S	97.2 (5.4)	100.0 (0.0)
PI 230976	S	80.4 (0.0)	96.5(6.6)
Woodruff	S	90.9 (13.0)	100.0 (0.0)
Tracy-M	R	0.0 (0.0)	0.0 (0.0)
(*Rdm1*/*Rdm2)*			
Crockett	R	0.0 (0.0)	0.0 (0.0)
(*Rdm3*)			
Dowling	R	0.0 (0.0)	0.0 (0.0)
(*Rdm4)*			
Hutcheson	R	0.0 (0.0)	0.0 (0.0)
(*Rdm4/Rdm5*)			
Benning	R	0.0 (0.0)	0.0 (0.0)
PI 398469	R	0.0 (0.0)	0.0 (0.0)
LSD (0.05)		**8.6**	**5.1**

***Data represent the average of 4 replicates per line with 12 plants per line in each replicate replicate).**
Means with the different letters are significantly different based on LSD (0.05)

Chapter 3

EVALUATION OF VIRULENCE OF *DIAPORTHE ASPALATHI* ON SOYBEAN CULTIVARS SUSCEPTIBLE TO STEM CANKER

Southern soybean stem canker is caused by the ascomycetous fungus *Diaporthe aspalathi* (syn. *Diaporthe phaseolorum* f. sp. *meridionalis*). This pathogen colonizes the vascular tissue of soybean stems and obstruct the translocation of water throughout the plant. The infection results in wilting, development of sunken, dark cankers on stems, vivid interveinal chlorosis, yield suppression, and in severe cases, plant death (Backman et al., 1985, Lalitha et al., 1988). Premature death of plants can result in a smaller seed as well as a lower amount of seed produced by the plant (Hildebrand, 1952; Fernandez et al., 1999).

Southern stem canker has been an economically important disease in the southeast United States (Krausz and Fortnum, 1983). However, it has not been as much of a problem in the midwest soybean growing areas of the U.S. In the mid 1980's, this disease rose to prevalence in the Southeast U.S., with an average yearly loss of 1.25×10^5 metric tons from 1985-1989 in soybean production. Southern losses reached a peak in 1989 at 2.02% of soybean production (Wrather et al., 1995). It should be noted that soybean yield suppression by stem canker over 28 states has recently been on the rise; with yield losses increasing from 1.8 million bushels in 1996 to 5.0 million bushels of soybean in 2007 (Wrather et al., 2009). Recently, combined losses to southern and northern stem canker

have reached levels of 12.7 million bushels in 2014 (http://extension.cropsciences.illinois.edu/fieldcrops/diseases/yield_reductions.php; verified 6 November 2016).

D. aspalathi is a necrotrophic pathogen that can overwinter on infected crop debris left in field. During the following growing season, inoculum may be spread from the crop debris to the current crop by rain or irrigation or high wind storms (Damicone et al., 1987). Conidia or ascospores that land on natural wounds such as leaf scars will germinate and initiate infection of the host (Ploetz and Shokes, 1987a; 1987b). Infection takes place early in the growing season, with symptoms presenting later in the season. Symptom development usually occurs once plants reach early reproductive stages, usually pod fill growth stage (R3) (Fehr et al., 1971).

Managing southern soybean stem canker typically focuses on reducing the inoculum levels and avoiding dispersal of inoculum (Backman et al., 1985). These preventative measures can be implemented in several different ways. Tillage regimes can bury infected stems and reduce the primary inoculum in the following season (Rothrock et al., 1985). Crop rotation using a non-host of stem canker can also deprive the organism of a nutrient source (Rothrock et al., 1988). Spread of stem canker can also be prevented by cleaning equipment used in an effected field before moving to a field with no history of stem canker (Backman et al., 1985).

Host resistance to southern soybean stem canker has proven to be the most effective form of disease control and prevention. The recurrence of the disease in the southeast U.S. in the 1980s suggests that host resistance might have been lost in commercial cultivars. Resistance to southern stem canker is conditioned by at least five distinct, dominant resistance genes designated as *Rdm1, Rdm2, Rdm3, Rdm4,* and *Rdm5*.

Resistance to *D. aspalathi* can be achieved by one resistance allele at any of these five loci (Ploetz and Shokes, 1987a). A resistance reaction is activated by these genes after infection, preventing the development of macroscopic symptoms, but not infection itself (Ploetz and Shokes, 1987a). These genes elicit an accumulation of phytoalexins in the plant, which is triggered after infection (Modolo et al., 2002).

D. aspalathi isolates differ in virulence in both susceptible and resistant soybean lines, with some isolates causing little to no disease (Keeling, 1985; Ploetz and Shokes, 1989; Pioli et al., 2003). This warrants investigation into the virulence of local isolates of *D. aspalathi* and subsequent suitability for use in evaluation of breeding lines for southern stem canker resistance. An isolate that causes low disease incidence or variable disease incidence among susceptible lines could give erroneous evidence of resistance for susceptible progeny in breeding programs.

The internal transcribed spacer 1 (ITS1) of DNA was selected for the molecular identification of fungal isolates collected from Georgian stem canker nurseries. Once a sequence was obtained for a collected isolate's ITS1 region, that sequence could be entered into a NCBI blast search. Highly similar ITS1 sequences from previously collected isolates can then be compared with the isolate in question. This section of fungal DNA is highly conserved for fungal species, and therefore ideal for isolate identification (Pryce et al., 2003).

The objective of this study was to characterize the virulence of isolates of *D. aspalathi* collected in Georgia to isolates obtained from Arkansas and the type isolate of D. aspalathi. This was achieved through the collection of fungal specimens from symptomatic soybean plants grown in UGA's stem canker nurseries located in Calhoun,

Bledsoe, and Plains, GA. Fungal isolates were purified and molecularly identified; those confirmed as *D. aspalathi* were then tested using susceptible soybean lines. Disease incidence, lesion length and progression, and days to plant death were utilized to assess and characterizing isolate virulence.

Materials and Methods

Collection *of Diaporthe aspalathi* Isolates

Southern soybean stem canker nurseries have been maintained in Georgia since 1984 for field screenings of soybean lines. A natural inoculum load has been built in these locations by allowing infected soybean debris to remain in field post-infection and harvest. The following season, breeding lines are then planted along with susceptible checks to test for field resistance to southern stem canker.

During the fall of 2014 and 2015, fungal isolates were collected from soybean stem canker nurseries in Bledsoe, Plains, and Calhoun, Georgia. Stem sections (30-40 cm long) were collected from symptomatic plants. Stems were then cut into ~5 cm pieces and sections were surface sterilized (one min in 70% ethanol, followed by two min in 10% sodium hypochlorite), removed and blotted dry. Individual pieces were placed onto acidified PDA. Fungal growth characteristic of *D. aspalathi*) was subsampled onto successive acidified PDA plates. These putative isolates had grey, tan, or white hyphal growth with and without pycnidial development (van Rensburg et al., 2006). Isolates were labeled according to nursery location, the order in which they were collected with the collection year included.

DNA was extracted from these fungal samples using the Chelex method (Walsh et al., 1991). A small amount of hyphae was added to 300 ml of 10% Chelex® solution.

Each solution was vortexed, centrifuged for 15 s, and then incubated for 20 min at 95^0C. Solutions were vortexed, centrifuged for 15 s and DNA recovered from the supernatant liquid. DNA concentration was diluted to 50 ng/ μL. PCR amplification of the internal transcribed spacer (ITS) was performed using primers ITS1 (sequence 5'-3' TCCGTAGGTGAACCTGCGG) and ITS4 (sequence 5'-3' TCCTCCGCTTATTGATATGC) (Bertini et al, 1999). The samples were heated at 94^0C for two min and 40 cycles were then performed at 94^0C for 30 s, 55^0C for 30 s, and 72^0C for 30 s. Samples were then held at 72^0C for five min. PCR product was checked for amplification and correct amplicon size (500-600 bp) by 1% agarose electrophoresis gel. Amplified product was purified using ExoSAPit reagent (Affymetrix, Santa Clara, CA) and sequenced at Georgia Genomics Facility using an applied Biosystems 3730xl 96-capillary DNA Analyzer (Thermo Fisher Scientific, Waltham, MA). Samples that yielded quality sequence of 200-550 bp were entered into the NCBI website blastn suite using the standard nucleotide blast search. Results of a 99% similarity or higher to ITS1 regions of *D. aspalathi* or *D. phaseolorum* f.sp. *meridionalis* in NCBI's nucleotide database were accepted as identification for fungal isolates.

A phylogenetic tree comparing the ITS1 regions of these *D. aspalathi* isolates to ITS1 regions other species of the *Diaporthe* genus was generated in Geneious (http://www.geneious.com, Kearse et al., 2012) to further confirm these isolates were not of any other *Diaporthe* genus. The cost matrix was set to 70%, and ITS1 regions from *D. caulivora, D. sojae,* and *D. longicolla* were included to be compared to the *D. aspalathi* isolates (Figure 3.2).

Seven isolates (CA13-13, CA10-13, BL39-14, BL22-14, BL3-15, NA-16, NB-16) were characterized in the experiment. Isolates CA10-13 and CA13-13 were collected from the stem canker nursery in 2013 in Calhoun, GA (Chapter 2). Isolates BL39-14, BL22-14, and BL3-15 came from the stem canker nursery in Bledsoe, GA in 2014 and 2015. In addition, two *D. aspalathi* isolates NA-16 and NB-16 were provided by Kimberly Rowe at University of Arkansas.

All these isolates of *D. aspalathi* were preserved by lyophilizing infected stem tissue. Working inoculum stock was maintained by subsampling isolates from potato dextrose agar (PDA) slants at 4^0C onto new acidified PDA every two to three weeks.

Inoculum Preparation and Inoculation Procedure

The modified inoculation method as outlined in Chapter 2 was utilized for the virulence assay. Specifically, toothpicks were cut in half, immersed in 500 mL oxgall liquid medium in 1 L flasks, and autoclaved for 20 min at 120^0C. The sterilized toothpicks were then arranged radially on oxgall agar in 9 cm petri plates. A plug of *D. aspalathi* from a two-week old culture on acidified PDA was placed in the center of each plate, and the fungus allowed to colonize the toothpicks for three weeks on a bench top at room temperature. Plants were inoculated at growth stage V2 between the cotyledon and first trifoliate. Petroleum jelly was applied to inoculation site and plants were placed in humidity chambers for 72 h post-inoculation. Plants were then removed from humidity chambers and placed on greenhouse benches.

Soybean Cultivars

Eight soybean cultivars/lines which are susceptible to southern stem canker were included in this experiment: Santa Rosa, Kirby, Hutton, Woodruff, G81-2057, Hartwig,

Bragg, and J77-339. These lines were chosen as they proved susceptible to virulent *D. aspalathi* isolates CA10-13, CA13-13, and DPM 1F in previous studies (Chapter 2).

Kord Presto (Riverhead, NY) sheet pots (10 cm x 10 cm) were arranged on the 12 border cells of 15 cell flats. The three middle cells were left vacant to prevent plant crowding and for simplified rating as used by Harris et al., 2015. Seed were planted in Jolly Gardener Pro-Line C/B growing mix (Atlanta, GA) with three seed per pot. The seedlings were thinned to two seedlings per pot at 7-10 days after planting. Each plant was staked using 36-inch stakes at two weeks after planting so that plant stems could withstand mechanical damage caused by the inoculation procedure. Seedlings were grown under 14 h of light per day and watered four to five times per week. They were also fertilized once a week with a Dosmatic (Hydro Systems Co., Carrollton, TX) model A30 dispenser set to deliver 200 μg mL^{-1} N from a stock of 20-20-20 Scotts Peters (The Scotts Miracle-Gro Company, Maryville, OH) water soluble fertilizer.

Experimental Design

A split plot design was used in this study. *D. aspalathi* isolate was designated as the whole plot factor, while soybean lines constituted a subplot factor. *D. aspalathi* isolates were replicated for eight times and each soybean line included once within each isolate replicate. Each soybean line in a replicate consisted of six seedlings grown in three pots. Each isolate replicate consisted of 48 seedlings planted into two 12-pot (24-seedling) flats. Thus, each isolate-soybean line combination was applied to 48 seedlings. The experiment was performed twice in the greenhouses at the University of Georgia Pathology greenhouses in Griffin, GA.

Data Collection and Analysis

Disease incidence and/or plant death due to disease were recorded at two, three, and four-weeks after inoculation. Lesion length on surviving, diseased plants was measured with a caliper and recorded for each seedling at two, three, and four weeks after inoculation. Missed plants due to poor germination or stem snapping were recorded as missing data. Days to plant death caused by each *D. aspalathi* isolate was recorded.

Results were analyzed using SAS Version 9.3 (SAS Institute, Carey, NC) Proc GLM. The number of plant death due to disease for each replicate was averaged for each *D. aspalathi* isolate and each soybean line at two, three, and four weeks after inoculation. The number of symptomatic plants for each replicate for each isolate and soybean line was also averaged at two, three, and four weeks post-inoculation. Mean of days to plant death was assessed by averaging the number of weeks that plants survived after inoculation for each isolate. Lesion length was also averaged for each replicate for isolate and each soybean line at two and three-weeks after inoculation. Lesion length was not analyzed at four-weeks after inoculation due to high amount of dead plants caused by the disease.

Results

Three isolates (BL22-14, BL39-14, and BL3-15) out of 141 fungal DNA samples submitted for sequencing, returned 99-100% similarity to the ITS sequences for *D. aspalathi* (or *D. phaseolorum* var. *meridionalis*) when entered into a NCBI Blast search. The usable length of sequences ranged from 213 bp (NA-16) to 510 bp (BL3-15). Isolates CA10-13 and CA13-13 were previously collected and identified in a similar manner (Table 3.2). Fungal isolates were identified based on 99% similarity of ITS

sequences to NCBI blast results. Isolates NA-16 and NAB-16 obtained from the University of Arkansas that was collected in Newport, AR, were confirmed as *D. aspalathi*. Forty-three percent of the total isolates returned 99% similarity of their ITS regions to those in the *Diaporthe/Phompsis* group (*D. sojae*, *P. longicola*, or *D. phaseolorum*), but were not *D. aspalathi* (Table 3.2). Eleven isolates returned high similarity scores to unidentified fungi.

No significant effect of planting dates in the greenhouse was observed on seedling death data ($P \geq 0.05$) (Table 3.3). Significant differences ($P<0.05$) were observed in seedling death among the seven isolates of *D. aspalathi* (Table 3.2 and 3.3). Significantly more seedlings were dead at two-, three- and four-weeks post-inoculation for *D. aspalathi* isolates, BL3-15 and BL22-15 compared to NA-16, NB-16, CA10-13, and CA13-13. Isolate BL3-15 caused objectively highest incidence of plant death at 25.7%, 63.4%, and 83.3% plant death at two, three and four weeks respectively. Isolate BL39-14 caused similar levels of plant death to the other Bledsoe isolates BL3-15 and BL22-15, as well as to the isolate CA10-13. *D. aspalathi* CA13-13 had the fewest dead seedlings at two, three, and four-weeks post-inoculation (5.4%, 25.2% and 61.9%) (Table 3.4).

Significant differences were also observed in the number of dead seedling among the soybean lines (Table 3.2 and 3.5). The fewest dead seedlings were observed with Bragg (8.4%, 34.7%, 64.9%) and Woodruff (7.0%, 33.6%, 57.6%)soybeans at each time point. Seedlings of G81-2057 (19.3, 64.0%, 83.3%) and J77-339 (21.1%, 57.7%, 78.8%) yielded the highest amount of dead seedlings for each time point. The percentage of dead seedlings at 4-weeks post-inoculation ranged from 57.6% (Woodruff) to 83.3% (G81-2057) for soybean lines at four weeks after inoculation.

Significant effects ($P<0.05$) of planting date were observed for percent disease (Table 3.6). The difference of disease incidence between planting dates was likely due to differences of the greenhouse environments (June, 2016). Disease incidence was reduced in the second planting date. Relatively high levels of disease incidence were caused by all of the tested isolates at two, three and four-weeks after inoculation. Of them, isolate BL3-15 conferred the highest levels of disease (99.7%) at four weeks after inoculation. Isolates CA10-13 and NB-16 caused lowest amounts of disease incidence at 95.3% at four-weeks post-inoculation (Table 3.7).

Days to plant death for all isolated were slightly significantly different between planting date ($P = 0.0479$) (data not shown). Average days to plant death ranged from 21 days to 26 days after inoculation till death. Isolate NB-16 had the shortest mean of days to plant death at 21 days after inoculation. All other isolates produced statistically similar means of days to plant death. It can be concluded that average days to plant death due to inoculation with these *D. aspalathi* isolates will fall between 21 and 24 days after inoculation.

There was a significant planting date effect on lesion length, but trends were similar between plant dates. The data from four-weeks after inoculation were not included in the analyses due to high numbers of plant death (and corresponding fewer numbers of lesions to measure). A significant difference was observed in lesion length among the seven isolates of *D. aspalathi* at two-weeks post-inoculation (data not shown). At two weeks after inoculation, isolates BL22-14 and BL3-15 had the longest lesion length at 42.8 mm and 41.8 mm, respectively. However, at three-weeks after inoculation, no significant differences in lesion length were observed among isolates. A significant

difference in lesion length at two-weeks, but not three-weeks, post-inoculation was also observed among soybean cultivars/lines (data not shown). Lesion length on Santa Rosa averaged 30.6 mm compared to 43.8 mm on G81-2057.

Discussion

This study has highlighted the necessity of molecular confirmation of the identity of fungal pathogens collected in field using ITS sequences. The ITS region of fungal DNA is highly conserved and, therefore, excellent for the molecular identification of fungal species (Pryce et al., 2003). We obtained sequences of usable quality for 141 fungal isolates from the Georgian stem canker nurseries, but only isolates CA10-13, CA13-13, BL39-14, BL22-14 and BL3-15 were confirmed as *D. aspalathi.* The sequences for these isolates were of variable usable length. One hundred and one the other isolates were molecularly confirmed as *Diaporthe sojae* and *Phomopsis longicolla* based on ITS sequences. The *Diaporthe* and *Phomopsis* species exhibited similar morphological growth habits (grey, white, or tan hyphal growth with or without pycnidial development) to isolates of *D. aspalathi* in culture. In addition, variability of morphological characteristics within isolates of *D.* characteristics was observed, especially under different growing conditions (Pioli et al., 2003). There is also the high possibility of the isolation secondary pathogens. For instance, the symptoms of stem blight caused by *D. longicolla* may be easily confused for stem canker, and leaf yellowing due to maturity may be confused with foliar symptoms of southern stem canker. *Diaporthe phasolourm* var. *sojae* is the causal agent of soybean stem and pod blight, a disease with which symptoms could be confused for soybean stem canker. The

symptoms for stem and pod blight often appear during reproductive stages of soybean growth, the same period when stem canker symptoms manifest (Lehman, 1923).

In the present study all the isolates of *D. aspalathi* were pathogenic, but differed slightly in virulence. Isolate BL3-15 was found to cause the highest disease incidence and seedling death. The other isolates collected were also highly virulent, making these fungal isolates ideal for use in screening soybean breeding lines for southern soybean stem canker resistance. The isolates collected from Newport, Arkansas (NA-16, NB-16) were considerably slower growing on media than the Georgian isolates tested, so this may have contributed to their reduced virulence. The hyphae may not have had time to colonize the toothpicks as thoroughly. Lower disease incidence observed with the Calhoun (CA10-13, CA13-13) isolates may have been due to environmental conditions in the greenhouse, as these rates of incidence were lower than ones observed in previous assays of these isolates (Chapter 2). Variability of the virulence of these isolates also indicates that they are potentially distinct from one another.

Differences among *D. aspalathi* isolates have been observed in previous studies as well. These isolates have been useful in assessing specific resistance or susceptibility reactions among soybean lines. Keeling (1985) observed significant differences in lesion length and plant death caused by isolates of *Diaporthe phaseolorum* var. *caulivora* (*D. aspalathi*) collected from Mississippi, Tennessee, and Ohio. For example, Isolate 81-102, collected from Tennessee, caused lesion length similar to that caused by several of the Mississippi isolates, but did not kill plants. There was also great variability in the lesion lengths (20.7-61.9 cm) and plant death produced (0-48% of plants dead) by the Mississippi isolates. Similarly, Ploetz and Shokes (1989) reported significant differences

in stem canker symptoms among vegetative compatibility groups when tested across different stem canker resistance backgrounds. The findings of these previous studies confirmed the necessity for characterization and confirmation of *D. aspalathi* isolates virulence. Isolates could potentially be avirulent, thus making them unsuitable for screening of stem canker resistance. Further testing using distinct isolates will be useful in confirming specific levels of soybean resistance or susceptibility to southern soybean stem canker.

It is important to screen soybean lines for southern stem canker resistance with locally collected isolates to detect and preempt a breakdown of currently deployed southern stem canker resistance genes. This phenomenon could occur due to pathogen adaptation to current resistance. Screening with endemic isolates is also an effective method for discovering new disease resistance sources that could be deployed in the event of pathogen adaptation to currently known resistance genes (McDonald and Linde, 2002). These isolates and isolates collected in Georgia in the future will be useful in screening of soybean breeding lines developed at the University of Georgia for resistance to southern soybean stem canker.

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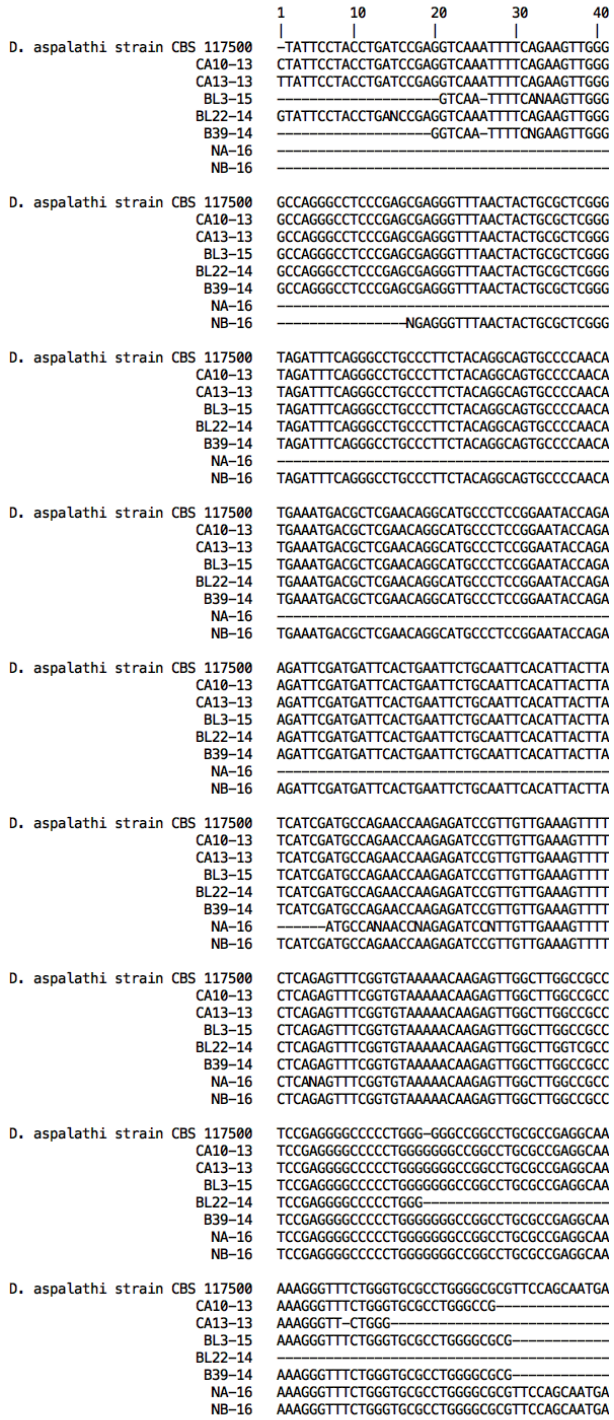

Figure 3.1. Sequence alignment of *D. aspalathi* CA13-13, CA10-13, B39-14, BL22-14, BL3-15, NA-16, and NB-16 ITS1 region with *D. aspalathi* strain CBS 117500 ITS 1 region.

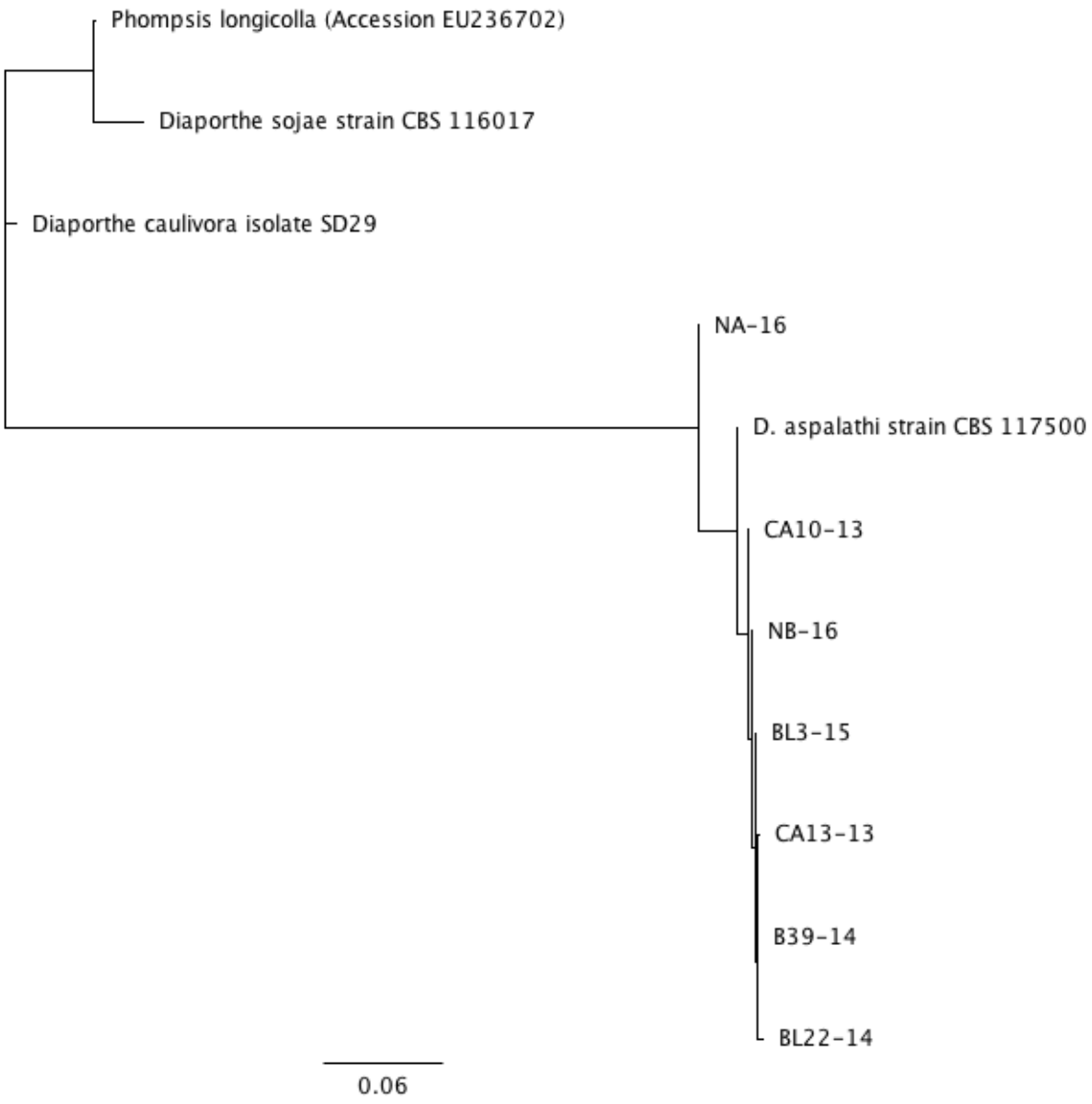

Figure 3.2. Phylogenetic comparison of ITS1 sequences of *D. aspalathi* isolates included in this study compared to other fungal species of the *Diaporthe.* The measure of support of 0.06 is represented by the reference bar.

Table 3.1. Similarity matrix displaying the similarity of the ITS1 regions of *D. aspalathi* isolates CA10-13, CA13-13, BL3-15, BL22-14, BL39-14, NA-16, NB-16 and *D. aspalathi* strain CBS 117500 used as reference

	D. aspalathi strain CBS 117500	CA10-13	CA13-13	BL3-15	BL22-14	B39-14	NA-16	NB-16
D. aspalathi strain CBS 117500		99.6	99.5	99.4	99.0	99.4	98.1	99.6
CA10-13	99.6		99.6	99.4	98.8	99.4	98.0	99.5
CA13-13	99.5	99.6		99.4	98.8	99.4	97.8	99.5
BL3-15	99.4	99.4	99.4		98.8	99.6	98.5	99.8
BL22-14	99.0	98.8	98.8	98.8		98.8	96.9	98.9
B39-14	99.4	99.4	99.4	99.6	98.8		98.5	99.8
NA-16	98.1	98.0	97.8	98.5	96.9	98.5		98.0
NB-16	99.6	99.59	99.58	99.83	98.9	99.83	98.01	

Table 3.2. Identification of fungal isolates collected from southern stem canker nurseries Bledsoe, Plains, and Calhoun, Georgia based on the NCBI blast of ITS sequence.

Species Name	Isolates Collected
Diaporthe sojae	49
Phomopsis longicolla	52
Diaporthe aspalathi	5
D. phaseolorum (not *meridionalis*)	8
Uncultured fungus/Unknown/Other	27
Total Isolates	141

*Isolates were identified based on a 96-99% similarity with NCBI blast of ITS sequence.

Table 3.3. Analysis of variance for plant death across soybean lines and *D. aspalathi* isolates at two, three and four weeks after inoculation.

Week	Source	DF	Mean Square	F Value	Pr > F
2	Planting Date	1	0.01	0.36	0.54
	Rep	7	0.01	0.59	0.75
	Isolate	6	0.33	10.73	<.0001
	Line	7	0.11	3.75	0.0006
	Isolate*Line	42	0.03	1.00	0.46
3	Planting Date	1	0.02	0.39	0.52
	Rep	7	0.06	0.97	0.44
	Isolate	6	1.00	14.53	<.0001
	Line	7	0.58	8.47	<.0001
	Isolate*Line	42	0.07	1.13	0.27
4	Planting Date	1	0.00	0.00	0.95
	Rep	7	0.09	1.54	0.15
	Isolate	6	0.40	6.76	<.0001
	Line	7	0.42	7.01	<.0001
	Isolate*Line	42	0.05	0.88	0.68

Table 3.4. The average number of dead seedlings for each isolate of *D. aspalathi* across eight susceptible soybean lines.

***D. aspalathi* Isolate**	2-Weeks After Inoculation	3-Weeks After	4-Weeks After Inoculation

	(Std. Dev.)	Inoculation (Std. Dev.)	(Std. Dev.)
	Seedling Death (%)*		
BL3-15	25.7 (23.8)	63.4 (23.7)	83.3 (17.9)
BL22-14	23.8 (21.1)	59.4 (29.9)	82.5 (20.4)
BL39-14	13.0 (18.8)	51.3 (29.4)	79.1 (25.3)
NB-16	11.0 (15.9)	43.8 (29.2)	70.8 (28.7)
NA-16	10.8 (15.2)	41.9 (28.3)	69.3 (29.5)
CA10-13	10.6 (16.3)	40.8 (30.5)	69.3 (26.8)
CA13-13	5.4 (11.5)	25.2 (24.4)	61.9 (28.8)
LSD (0.05)	6.1	9.1	8.5

*Data represent the average of 16 replicates for each treatment with 24 plants per replicate (384 plants total).

Table 3.5. The average number of dead seedlings for each susceptible soybean line across seven isolates of *D. aspalathi*.

Stem Canker Susceptible	2-Weeks After Inoculation	3-Weeks After Inoculation	4-Weeks After Inoculation

Soybean Line	(Std. Dev.)	(Std. Dev.)	(Std. Dev.)
		Seedling Death (%)*	
J77-339	21.1 (23.2)	57.7 (28.7)	78.8 (20.7)
G81-2057	19.3 (20.2)	64.0 (27.1)	83.3 (18.1)
Hartwig	17.8 (22.4)	52.1 (30.4)	81.2 (21.7)
Kirby	14.6 (17.9)	41.9 (28.9)	69.8 (27.1)
Santa Rosa	13.6 (18.1)	42.4 (26.2)	71.2 (27.4)
Hutton	13.2 (18.4)	48.1 (31.1)	78.0 (25.0)
Bragg	8.4 (14.4)	34.7 (28.9)	64.9 (30.3)
Woodruff	7.0 (12.9)	33.6 (28.4)	57.6 (30.7)
LSD (0.05)	6.5	9.7	9.1

*Data represent the average of 16 replicates per treatment with 24 plants per replicate (384 plants total).

Table 3.6. Analysis of variance for disease incidence across soybean lines and *D. aspalathi* isolates at two, three and four weeks after inoculation.

Week	Source	DF	Mean Square	F Value	Pr > F
2	Planting Date	1	1.244	35.65	<.0001
	Rep	7	0.016	0.48	0.84
	Isolate	6	0.151	4.34	0.0002

	Line	7	0.070	2.02	0.05
	Isolate*Line	42	0.031	0.91	0.63
3	Planting Date	1	0.272	18.41	<.0001
	Rep	7	0.004	0.29	0.95
	Isolate	6	0.034	2.36	0.03
	Line	7	0.024	1.63	0.12
	Isolate*Line	42	0.011	0.76	0.86
4	Planting Date	1	0.240	25.61	<.0001
	Rep	7	0.004	0.43	0.88
	Isolate	6	0.016	1.78	0.10
	Line	7	0.010	1.14	0.33
	Isolate*Line	42	0.007	0.80	0.81

Table 3.7. The average disease incidence caused by seven isolates of *D. aspalathi* across eight susceptible soybean lines.

***D. aspalathi* Isolate**	2-Weeks After Inoculation (Std. Dev.)	3-Weeks After Inoculation (Std. Dev.)	4-Weeks After Inoculation (Std. Dev.)
	% Disease Incidence		
BL3-15	93.6 (2.5)	98.7 (1.5)	99.7 (1.3)
BL22-14	95.5 (2.4)	99.4 (1.6)	99.4 (1.2)

BL39-14	87.7 (2.5)	95.8 (1.5)	97.3 (1.2)
NB-16	85.5 (2.3)	92.8 (1.5)	95.3 (1.2)
NA-16	86.3 (2.5)	97.5 (1.6)	97.7 (1.3)
CA10-13	80.9 (2.4)	93.2 (1.6)	95.3 (1.2)
CA13-13	87.5 (2.3)	96.8 (1.6)	97.3 (1.2)
LSD (0.05)	6.4	4.2	3.3

*Data represent the average of 16 replicates with 24 plants per replicate (384 plants total) for each isolate.

Chapter 4

SUMMARY

Southern stem canker of soybean, caused by *Diaporthe aspalathi* (syn. *Diaporthe phaseolorum* f.sp. *meridionalis*), inflicted large losses to growers in the southeastern United States in the 1980s. Southern stem canker starts as small, reddish-brown lesions extending from leaf scars, wounded tissue, or leaf nodes during early reproductive stages of the soybean. As infection progresses and the plant reaches growth stage R5, the affected tissue transforms into sunken, unilateral necrotic cankers that will block vascular tissues. Severe infection can reduce yield and kill plants. By far, the most effective method for management of soybean stem canker is to use resistant soybean cultivars. Resistance to *D. aspalathi* in soybean is controlled by at least five major, dominant genes: *Rdm1*, *Rdm2*, *Rdm3*, *Rdm4* and *Rdm5*. Field screening for stem canker resistance is labor intensive and requires an entire season. A relatively quick greenhouse assay that produces consistent disease phenotype would facilitate germplasm screening for stem canker resistance.

A toothpick inoculation method of Keeling (1982) and modified by Ploetz an Shokes (1987) for southern stem canker was modified to increase disease consistency. Briefly, Briefly, inoculum was prepared on oxgall agar and toothpicks were soaked and autoclaved in oxgall broth prior to plating. Inoculation was performed at growth stage V2 between the cotyledon and the first trifoliate. Inoculation sites were sealed with petroleum jelly and seedlings were placed in humidity chambers for 72 h post inoculation. Stem canker disease was not observed on the six resistant soybean lines as expected. By four weeks post-inoculation there was no significant difference between diseases caused by the three isolates of *D. aspalathi* (CA13-13: 99.1% of plants with disease; CA10-13: 99.0%, and DPM 1F: 98.7%) averaged across all susceptible lines.

Differences in virulence among isolates of *D. aspalathi* has been reported. For example, significant differences were observed in lesion length and plant death caused by isolates of *Diaporthe phaseolorum* var. *caulivora* (*D. aspalathi*) collected from Mississippi, Tennessee, and Ohio (Keeling, 1985). Ploetz and Shokes (1989) observed significant differences in stem canker symptoms among vegetative compatibility groups when tested across various levels of stem canker resistance. In the present study seven isolates of *D. aspalathi* were pathogenic on eight susceptible soybean lines, but differed slightly in virulence. Isolate BL3-15 was found to cause the highest disease incidence and seedling death. The other isolates collected were also highly virulent, making these fungal isolates ideal for use in screening soybean breeding lines for southern soybean stem canker resistance.

The modified toothpick assay produced in the present study results in consistent stem canker disease phenotype on susceptible soybean lines. The greenhouse assay requires 2-week old fungal inoculum grown on toothpicks with seedlings at V3 stage of growth. Disease can be assessed from 3 to 4 weeks after inoculation. The assay, combined with a small collection of virulent *D. aspalathi*, will enable identification and localization of new sources of resistance to southern stem canker.