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MORPHOLOGICAL, CULTURAL, MOLECULAR, AND PATHOLOGICAL CHARACTERIZATION OF PHOMOPSIS SPECIES OCCURRING ON COTTON AND SOYBEAN

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Abstract

Phomopsis species were isolated from cotton and soybean tissues in the southeastern United States. Fungal isolations determined *Phomopsis longicolla* and *P. phaseolina* colonized roots, petioles, leaves, and pod tissues of soybean, whereas a *Phomopsis* sp. was isolated from roots, petioles, leaves, and boll tissues of cotton. Morphological and cultural differences were found between the cotton *Phomopsis* isolates and the two soybean pathogens, *P. phaseoli* and *P. longicolla.* Toothpick inoculations determined that all three species produced symptoms on cotton and soybean. Symptoms were more severe for each species on the primary host. Restrictive fragment length polymorphism analyses of polymerase chain reaction (PCR) fragments were used to distinguish *Phomopsis* isolates from soybean and cotton. Specific primers were used to amplify a 550 base pair sequence known as the Internal Transcribed Spacer (ITS) Region, a mitochondrial rRNA gene. PCR fragments from forty-nine *Phomopsis* isolates were sequenced. DNA sequence analyses displayed distinct variation between *Phomopsis* isolates from cotton and soybean. This is noteworthy because the naming of *Phomopsis* species thus far has been strongly based on plant hosts.

Introduction

The Diaporthe/Phomopsis complex has a long history due to the economic importance of these species. Despite its importance, ubiquity, and abundance, no extensive compilation or taxonomic work concerned with *Phomopsis* has been published since Grove's treatment of the group with stem and leaf fungi in Britain (1935). *Phomopsis* species isolated from cotton and soybean tissues have been observed in the southeast and mid-south. *Phomopsis phaseoli* and *P. longicolla,* both pathogenic on soybean, are well described in the literature. Ivey and Pinckard described *Phomopsis gossypii* as the causal agent of a blossom rot of cotton. However, no description of the fungus was included in the abstract. Molecular techniques are currently being used to aid in the identification of *Phomopsis* species. Objectives of the current study are to characterize isolates of *Phomopsis* species from cotton and soybean using morphological, cultural, molecular, and pathological techniques in order to differentiate between isolates of *Phomopsis* from cotton with those from soybean.

Materials and Methods

Field Isolations

Isolates of *Phomopsis* used in this study were obtained from soybean and cotton tissues collected from plants in Louisiana, Mississippi, and Alabama. Immediately after plants were collected, they were sealed in plastic bags and stored on ice prior to examination at Auburn University. After collecting plant samples were washed in running tap water for 15 min then one 5-mm section was excised from the roots, petioles, and leaves of each plant. Cotton boll and soybean pod tissues were also included. Plant tissues were surface sterilized in 95% ethanol followed by 0.10% sodium hypochlorite (NaOCl). Surface sterilized roots, petioles, leaves, bolls, and pods were then aseptically plated on acidified potato dextrose agar (APDA). The plates were incubated at 25 C for 3 to 10 days. *Phomopsis* isolates were selected from the mycelium growing out from the plant tissues. All isolates were maintained on Difco potato dextrose agar (PDA) slants.

Morphological, Cultural, and Pathological Studies

For the growth studies, mycelial plugs 3 mm in diam taken from 4 d old *Phomopsis* cultures growing on PDA were placed in the center of 100 mm Petri dishes. Cultures were grown on PDA at 25 C in the dark for eight days. Colony diameter measurements in mm were obtained at 72 h, and 144 h. Each isolate was replicated 4 times. Pathogencity studies were conducted following the aseptic tooth pick inoculation of 3 week old soybean and cotton seedlings. Each experimental unit was replicated five times in a randomized complete block design.

Nucleic Acid Extraction and PCR Amplification

Mycelia were grown on Difco potato dextrose broth for four days at room temperature under ambient light conditions. Mycelia were harvested by vacuum filtration on Whatman No. 1 filter paper and freeze-dried prior to DNA extraction. DNA was extracted with the Dneasy Plant Mini Kit (Qiagen Inc., Chatsworth, California) according to the manufacturer's instructions using approximately 15 mg dried tissue. The internal transcribed spacer (ITS) regions 1 and 2, including the 5.8S rDNA, were amplified in 50 µL reactions on a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, California) under the following reaction conditions: 10-15 ng of genomic DNA, 200 mM each dNTP, 2.5 units Amplitaq Gold (Applied Biosystems, Foster

City, California), 25 pmoles each of primers ITS5 and ITS4 (White et al., 1990) and the supplied 10X PCR buffer with 15 mM $MgCl_2$. The thermal cycler program was: 10 min at 95 C followed by 35 cycles of 30 s at 94 C, 30 s at 55 C, and 1 min at 79 C, with a final expression period of 10 min at 72 C. After PCR amplification, the products were purified with QIAquick columns (Qiagen Inc., Chatsworth, California) according to the manufacturer's instructions. The resulting amplified products were sequenced at Auburn University Genomics facility. Sequences were edited using Vector NTI Suite.

Results and Discussion

Fungal isolations determined *Phomopsis longicolla* and *P. phaseolina* colonized roots, petioles, leaves, and pod tissues of soybean, whereas a *Phomopsis* sp. was isolated from roots, petioles, leaves, and boll tissues of cotton. Morphological and cultural differences were found between the cotton *Phomopsis* isolates and the two soybean pathogens, *P. phaseoli* and *P. longicolla* (Table 1). Toothpick inoculations determined that all three species produced symptoms on cotton and soybean. Symptoms were more severe for each species on the primary host. DNA sequence analyses displayed distinct variation between *Phomopsis* taken from cotton and from soybean. This is noteworthy because the naming of *Phomopsis* species thus far has been strongly based on the plant host. The Phomopsis isolate obtained from cotton tissues in this study was compared to the type species Phylctaena gossypii Sacc., however the herbarium material was not in good condition. Further evaluation is needed with existing specimens, specifically *Phomopsis gossypii* (Sacc.) Montegut.

Table 1. Characteristics of three *Phomopsis* species occurring on cotton and soybean.

	P. phaseoli	***P. longicolla***	***Phomopsis* sp.**
Colony			
Color	white to dark brown with age	white occasionally greenish yellow	white occasionally greenish yellow
Texture	floccose, ropy, pulvinate stromata	floccose, dense, large stromata	floccose, dense, scattered stromata
Pycnidial beaks	$\leq$ 200 um	200-500 um	$\leq$ 200 um
Growth			
72 hr	49 mm	32 mm	39 mm
144 hr	entire plate	46 mm	entire plate
Symptoms			
Cotton	mild	mild	moderate to severe
Soybean	moderate	moderate	mild