MISSISSIPPI SOYBEAN PROMOTION BOARD
PROJECT NO. 60-2014 (YEAR 2)
2014 Final Report

Project Title: Characterization of Endophytic Microbial Communities Associated with the Soybean Charcoal Rot Disease System

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EXECUTIVE SUMMARY

Charcoal rot caused by the fungal pathogen *Macrophomina phaseolina* is one of the most economically important soybean diseases in the South. Some healthy soybean plants can always be found in disease patches where almost all plants died of charcoal rot disease.

Specific objectives of the project are 1) Characterization of endophytic bacterial and fungal communities associated with the soybean-charcoal rot disease system using both culture-dependent and independent analyses; and 2) Investigation of the effects of inoculation of bacteria and/or fungi on disease development and soybean growth.

Research Activities and Achievements

Plant sample collection: Four pairs of diseased and healthy plants were collected from patches affected by charcoal rot in Leflore County in 2013 and 2014. Four additional asymptomatic plants were collected from areas of the same field where no symptoms of charcoal rot disease were evident. Plants were used for fungal and bacterial endophyte isolation and profiling of microbial communities using Illumina sequencing.

Bacterial isolation and profiling of bacterial communities: Bacterial endophytes were isolated using a centrifugation-based protocol. In total, 6,865 representative colonies of bacteria were collected and stored in ultralow freezer. The remaining bacteria were washed from the plate and stored in ultralow freezer for DNA extraction. Each sample of DNA is currently being PCR-amplified using barcoded 16S rDNA primers. The resulting products will be sent to a commercial laboratory for Illumina sequencing, which will be used for analysis of bacterial communities. We anticipate receipt of this sequencing data mid-summer, 2016.

Fungal isolation and profiling of fungal communities: Fungal endophytes were isolated from plant tissues and grouped by morphological similarities. PCR-based identification of culturable fungal isolates has been completed from all samples. DNA samples were shipped to a commercial laboratory for Illumina sequencing. This sequencing procedure failed. We are currently repeating the work using a different protocol and anticipate receiving the Illumina data in mid-summer, 2016.

Bioinformatic analysis of Illumina data: These data will provide characterization of the uncultured and cultured bacterial and fungal endophyte communities and will also be used to determine the best culture conditions of various endophytes.

Evaluation of antifungal activities of bacteria and fungi found in healthy plants: A total of 6,865 isolates of cultured bacteria and over 1000 isolates of cultured fungi were stored at -80^{o}C for use in this study. A preliminary plate bioassay of each isolate is done by co-culturing the bacterial or

fungal isolate on a plate with the charcoal rot pathogen (strain 151). As of April 25, 2016, we have found and identified 28 bacterial isolates and 21 fungal isolates that showed the activity against the charcoal rot pathogen. To date approximately 35% of the bacterial isolates and 30% of the fungal isolates have been assayed. Upon completion of all plate bioassays, some of the isolates will be selected for use in greenhouse studies.

Impacts and Benefits to Mississippi Soybean Producers: This work will lead to a better understanding of the endophytic microbial communities present in soybean plants and discovery of the microbial isolates that may help plants resist the disease.

End Products — Completed or Forthcoming

There have been no outputs from this project to date. We anticipate publication of three papers in referred scientific journals with one published within the next year.

BACKGROUND AND OBJECTIVES

Charcoal rot caused by the fungal pathogen *Macrophomina phaseolina* is one of the most economically important soil-borne soybean diseases in the southern US. No practical management approaches or resistant varieties are available for management of the disease. Some asymptomatic soybean plants can always be found in disease patches where almost all plants died of charcoal rot disease. However, these plants have the same genetic makeup, grow under the same soil and weather conditions, and have the same pathogen pressure.

Specific objectives of the project are 1) Characterization of endophytic bacterial and fungal communities associated with the soybean-charcoal rot disease system using both culture-dependent and independent analyses; and 2) Investigation of the effects of inoculation of bacteria and/or fungi on disease development and soybean growth.

REPORT OF PROGRESS/ACTIVITY

Objective 1: Characterization of endophytic bacterial and fungal communities associated with the soybean-charcoal rot disease system using both culture-dependent and independent analyses.

Figure 1. Asymptomatic and diseased (arrow) plant pair collected for isolation of bacterial and fungal endophytes.

On September 2, 2013 and again on September 7, 2014 four pairs of diseased and asymptomatic plants were collected from patches of plants affected by charcoal rot in a soybean field in Leflore County near Sidon, MS (Figure 1). Four additional asymptomatic plants were collected from areas of the same field where no symptoms of charcoal rot disease were evident. In 2013, a soil sample was collected from the root zone of each plant and sent to the Extension Plant Disease and Nematode Diagnostic Laboratory at Mississippi State University for nematode analysis. Soil from the rhizosphere of only one plant had a plant parasitic nematode present at greater than threshold levels. That plant was one of the four asymptomatic plants from a portion of the field where charcoal rot disease was not evident.

Plants were processed for fungal and bacterial endophyte isolation. Each plant was washed with tap water to remove soil and other contaminants from the surface. The plant was then surface disinfected by submerging it for five minutes in a 10% bleach solution prepared with sterile water. When present, leaves of the plants were submerged only one minute to prevent decomposition in the bleach solution. The entire plant was then soaked in two five-minute rinse baths of sterile water to remove residual bleach. Each plant was transferred to a sterile surface in a laminar flow hood and allowed to air dry. The plant was halved longitudinally using flame-sterilized pruners and sterile scalpels. One half of the plant was processed for isolation of bacterial endophytes and the other half was used for isolation of fungal endophytes.

Bacterial endophytes were isolated using an enrichment protocol as modified from Ikeda, et al (2009). The plant half for bacterial extraction was cut into small pieces using sterile instruments and placed into sterile buffer in a sterile blender jar. The contents were processed at high speed three times at one-minute each with three-minute ice bath incubation between processing periods. The macerated plant tissue and buffer were filtered through Miracloth® and centrifuged to remove plant tissues. The subsequent bacterial suspension was filtered through Kimwipes® and centrifuged twice more to concentrate the bacteria. Finally, bacteria were isolated from remaining plant cells and concentrated on a Nycodenz® density-gradient by ultracentrifugation. The layer of concentrated bacteria (Figure 2) was removed from the gradient, cleansed of the Nycodenz and suspended in 2mL of 50mM Tris-HCl, pH 7.5 buffer. A portion of the final bacterial suspension was used to prepare serial dilutions to 10^{-8} in sterile water. The remainder of the suspension was stored at -80^{o}C and later used as the uncultured bacterial specimen for extraction of total genomic DNA (gDNA). The serial dilutions were plated onto two different media: glucose-yeast extract agar (Watanabe and Barrauio, 1979) and 0.5X R2A agar. The 0.5X R2A agar was prepared by combining 9.2g Difco R2A Agar (Becton Dickinson #218263) and 9.6g Difco Granulated Agar (Becton Dickinson #214530) per liter. Two plates of each medium-dilution combination for the 10^{-1}, 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8} dilutions were incubated at 28^{o}C in each of three environments: aerobic, microaerophilic and anaerobic. The microaerophilic and anaerobic environments were achieved using BD GasPakEZ systems (Becton Dickinson 260001 and 260680, respectively).

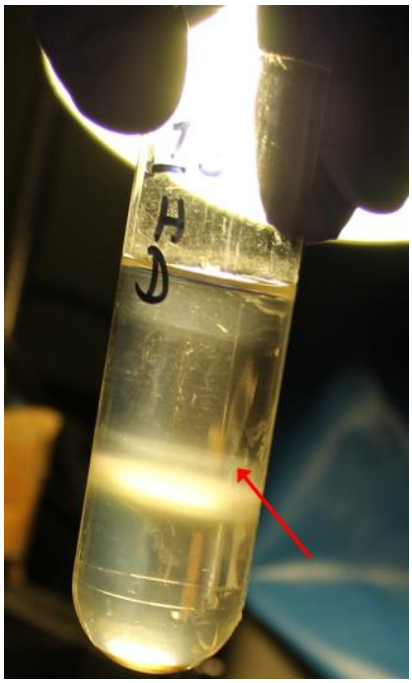

Figure 2. Nycodenz density gradient after ultracentrifugation**.** Arrow indicates band of concentrated bacteria from a single plant. Arrow indicates the band of concentrated bacteria isolated from a single plant.

Plates were examined at seven-day intervals and incubation terminated when bacterial growth covered 25% or more of the plate or when the medium in the plate began to desiccate (Figures 3-5). A total of 6,865 representative colonies of bacteria were picked up from the plates and stored in 20% glycerol at -80^{o}C for later use in identification and bioassays. The remaining bacteria were washed from the plate using sterile water. The plate washes were stored at -80^{o}C for gDNA extraction from each sample at a later date

In each year, total gDNA from each uncultured sample and from each medium-oxygen environment combination of cultured bacteria was extracted using the MoBio Power Soil DNA Isolation Kit (MoBio #12888). Extraction of gDNA from the uncultured samples was done in a single reaction per sample whereas extractions for the cultured samples were done in triplicate. Modification of the kit protocol to accommodate these samples was made as follows. Each 2mL tube of plate wash was centrifuged at 10,000Xg for 3 minutes to pellet the bacteria and the supernatant was discarded. The liquid from the PowerSoil bead tube was used to resuspend the bacterial pellet, transferred back into the bead tube, and the standard kit protocol was followed thereafter. Each sample of gDNA is currently being PCR-amplified using barcoded 16S rDNA primers (Kozich, et al 2013). The resulting products from all reactions will be combined in equimolar concentration and will be sent to a commercial laboratory for Illumina sequencing. We anticipate receipt of this sequencing data mid-summer, 2016. The sequencing results will

provide characterization of the uncultured and cultured bacterial endophyte communities of the plants and will also be used to determine the best growth medium-oxygen environment for culture of various bacterial endophytes. Comparison of the endophyte communities of asymptomatic and diseased plants as determined by the Illumina sequencing will lead to a better understanding of the potential role of the various bacterial isolates with regard to whether charcoal rot disease develops.

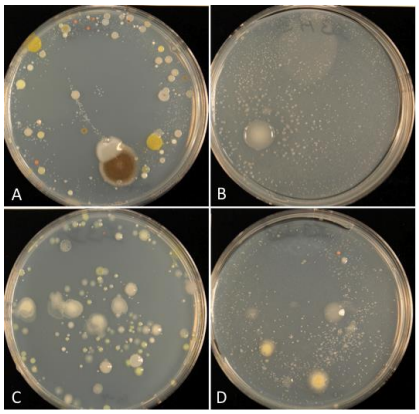

Figure 3. Aerobic bacterial endophytes from asymptomatic (A, B) and diseased (C,D) plants depicted in Figure 1. The two growth media are R2A (A, C) and GYE (B, D).

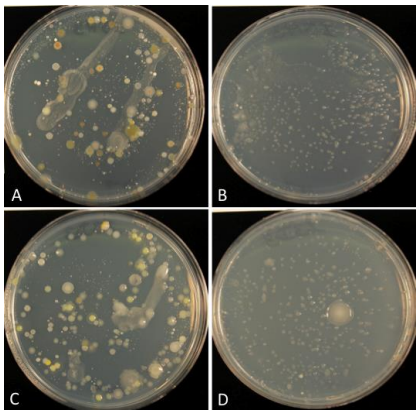

Figure 4. Microaerophilic bacterial endo-phytes from asymptomatic (A, B) and diseased (C, D) plants depicted in Figure 1. The two growth media are R2A (A, C) and GYE (B, D).

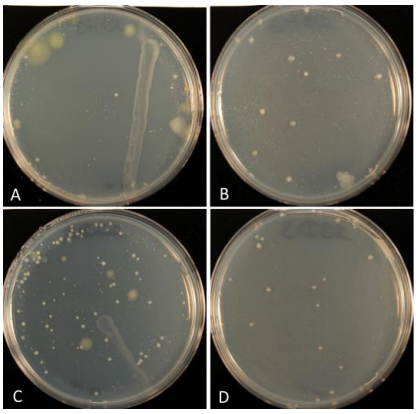

Figure 5. Anaerobic bacterial endophytes from asymptomatic (A, B) and diseased (C,D) plants depicted in Figure 1. The two growth media are R2A (A, C) and GYE (B, D).

Fungal endophytes were isolated from each plant part (root, stem leaf) separately. Each plant part was washed for 30 seconds in 70% ethanol solution, rinsed in sterile water for 30 seconds, and allowed to air dry. Sections (1-cm) were cut from the tissue using a sterile scalpel. Three sections of a tissue were placed onto plates containing potato dextrose agar (PDA), and another three pieces were placed onto plates containing water agar (WA). Three sets of plates were prepared and incubated in the three different oxygen environments used for bacterial incubation.

Plates were incubated in the dark at 28^{o}C for 48 to 72 hours, at which time emerged mycelium was subcultured onto sterile plates containing the appropriate medium and incubated in the same environment as the original isolation plate for approximately 10 days. Plates were initially grouped by morphological similarities (color, texture, pattern of growth, etc.). DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Inc.) and quantified on 1% agarose electrophoresis gel and NanoDrop spectrophotometer. The ITS region was then amplified using primers ITS1f and ITS4 and the amplified product sent to a commercial laboratory for sequencing. Identification of culturable fungal isolates has been completed from all samples and is summarized in Table 1. The most commonly occurring species are *Phoma* sp., *Penicillium chryseogenum*, *Diaporthe phaseolorum*, *Fusarium* sp., *Aspergillus flavus*, *Diaporthe sojae*, *Fusarium oxysporum*, *Trichoderma reesei*, *Hypocrea lixii*, and *Alternaria alternata*.

Table 1. Percent Isolation of Culturable Endophytic Fungi From Soybean Plant Tissues

Fungi	Summary of 2013 and 2014 Percent Isolations from Soybean Plant Tissues											
	Aerobic				Anaerobic				Microaerobic			
	Healthy Plants		Diseased Plants		Healthy Plants		Diseased Plants		Healthy Plants		Diseased Plants	
	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014
Alternaria sp.	45%	10%	57%	10%	20%	10%	40%	8%	38%	7%	50%	3%
Alternaria alternata	0	5%	0	0	0	5%	0	3%	0	7%	0	8%
Cladosporium sphaerospermum	20%	8%	25%	1%	10%	9%	8%	0	18%	5%	20%	8%
Cladosporium cladosporioides	22%	0	25%	0	9%	0	10%	0	15%	0	18%	0
**Fusarium solani*	30%	11%	24%	2%	37%	7%	25%	14%	32%	4%	28%	5%
**Fusarium chlamydosporum*	25%	15%	28%	12%	20%	18%	25%	21%	20%	14%	25%	7%
Clonostachys rosea	18%	10%	20%	19%	15%	10%	15%	15%	20%	10%	20%	13%
**Fusarium oxysporum*	0	7%	0	0	0	3%	0	1%	0	4%	0	8%
Phoma sojicola	4%	9%	0	9%	0	2%	0	0	0	9%	0	11%
Phoma glomerata	0	3%	0	12%	0	20%	0	13%	0	9%	0	11%
Trichoderma harzianum	18%	6%	11%	8%	2%	3%	12%	4%	18%	9%	11%	9%
Trichoderma sp.	9%	10%	12%	23%	5%	7%	10%	19%	--	10%	4%	10%
Verticillium lecanii	1%	2%	2%	0	0	0	0	1%	1%	5%	4%	0
Verticillium sp.	0	1%	0	2%	0	3%	0	0	0	3%	0	4%
Cultured fungi (no GenBank match)	2%	3%	4%	2%	19%	3%	21%	1%	6%	4%	5%	3%

**Fusarium* spp. are based on transcription elongation factor (TEF-1α) and internal transcribed spacer data as compared to FUSARIUM-ID database and morphological information.

Preparation of fungal samples for Illumina MiSeq barcoding analysis begins with the tissue samples stored at -80 C at the original times of sampling (2013 and 2014). These samples were dissected adjacent to tissue pieces that were plated on selective media to produce the initial culturable fungal isolates, and stored at -80 C for this use. DNA was extracted using MoBio PowerPlant Pro DNA Isolation. Extracted DNA was quantified using a NanoDrop 1000 Spectrophotometer and viewed on a 1% agarose rapid electrophoresis gel. Extractions were repeated if the quality of the DNA was judged to be too poor for PCR use, and many of the stem and root tissues fell into this category. Samples were then subjected to an initial PCR using ITS1F and ITS4 to select only the ITS portion of the genome for further use. After PCR, the DNA was again viewed on a 1% agarose rapid electrophoresis gel, and quantified using a NanoDrop 1000 spectrophotometer. This portion resulted in approximately 72 samples of clean, amplified DNA. Additionally, DNA from 237 samples of culturable fungal isolates was selected to be included in the pooled sample to represent the culturable portion of the fungal community. Samples were then taken through a cleanup procedure using AmPure paramagnetic beads to remove excess oligos, nucleotides, and other remnants of previous procedures. Following cleanup, a second PCR reaction was completed on each sample with ITS1F and barcoded ITS4 primers. Samples were visualized on a 1.5% agarose electrophoresis gel. Once the barcoded

primer was attached to each sample, there remained only a series of AmPure cleanups, quantification of DNA, and pooling of the samples. At this stage, a failure of the AmPure bead cleanups occurred, and amplified DNA from multiple samples was lost during the cleanup process

After weeks of troubleshooting this procedure, adjustments were made that accommodated failures with the bead protocol in cleaning up the small, barcoded segments of DNA. Qiagen QIamp PCR column-based cleanup was substituted at this stage to recover the highest quality of barcoded DNA, and samples were quantified on a NanoDrop 1000 spectrophotometer. Samples were pooled at 100 ng/sample into one 1.5-mL tube. One tube of the prepared DNA was shipped to Eurofins MWG Operon for sequence analysis on the Illumina MiSeq platform. This sequencing procedure failed, possibly due to the length of the barcoded primers used. We are currently repeating the work from the initial PCR reactions onward. We are using a different protocol as described by Veach et al (2015) and anticipate receiving the Illumina data in mid-summer, 2016.

Bioinformatic analysis of Illumina data for both the bacterial and fungal communities will be accomplished as described by Veach et al (2015).

Objective 2: Investigation of the effects of inoculation of bacteria and/or fungi on disease development and soybean growth.

During work for Objective 1 a total of 6,865 isolates of cultured bacteria and over 1000 isolates of cultured fungi were stored in 15-20% glycerol at -80^{o}C so that they would be available for use in this portion of the study. Plate bioassays are being used to determine which isolates may be involved in charcoal rot disease suppression or resistance. Each isolate was removed from storage and cultured using the media and environmental conditions in which it was initially isolated. The recovered isolates were used for standardized plate bioassays. On removal from -80^{o}C freezer, the bacterial isolates are streaked onto NBY medium and fungal isolates are plated onto PDA.

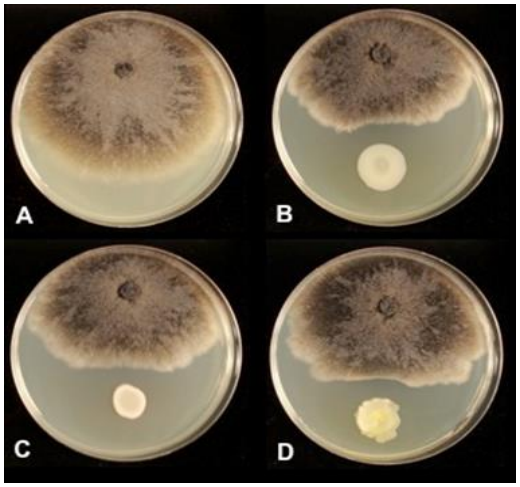

Figure 6. Inhibition of endophytic bacteria isolated from asymptomatic soybean plants to *Macrophomina phaseolina* 151. An agar disc containing the isolate 151 was inoculated onto PDA plates and a drop of bacterial isolates containing approximately 1000 cells was inoculated on the plates. The inoculated plates were incubated for 4 days. A: Control; B: 13SE-125; C: 13SE-121; and D: 13SE-36.

After sufficient growth, a preliminary plate bioassay is done by co-culturing the bacterial or fungal isolate on a plate with *M. phaseolina* 151. Those isolates showing activity against *M. phaseolina* 151 are then used for a quantified plate bioassay. Bacterial isolates are inoculated into nutrient broth yeast extract (NBY) broth and incubated overnight at 28^{o}C with shaking. The culture is centrifuged to pellet the bacteria, which are then washed with sterile isotonic buffer. The bacteria are resuspended in sterile buffer and approximately 1000 cells are pipetted in a small drop onto plates of NBY agar and PDA. An agar disc (5mm diameter)

from a culture of *M. phaseolina* 151 is simultaneously placed 2cm from the bacterial inoculum. Plates are inverted and incubated at 28°C for 4 days, then observed to determine whether the bacterial isolate inhibited the *M. phaseolina* 151 (Figure 6). Bacterial isolates demonstrating the ability to inhibit *M. phaseolina* 151 are then tentatively identified using PCR of the 16S rDNA. In an effort to streamline this screening and identification process, we are now screening all isolates and will complete the PCR identifications after all screening is done. This will allow large batches of PCR to be accomplished and thereby save considerable time. To date approximately 35% of the bacterial isolates from 2013 and 2014 have been assayed. Representative data from those isolates that have been sequenced are presented in Table 2.

Table 2. Bacterial isolates displaying antifungal activity against *M. phaseolina* 151.

Isolate	Identity	Activity Reported in Literature	
		Antifungal	Antibacterial
13SE-6B1	*Bacillus subtilis*	X	
13SE-6B2	*Bacillus tequilensis*	X	X
13SE-36	*Pantoea dispersa*	X	X
13SE-37	*Pseudomonas oryzihabitans*	X	
13SE-38	*Pantoea dispersa*	X	X
13SE-116	*Pseudomonas geniculata*	X	
13SE-117	*Pseudomonas geniculata*	X	
13SE-121	*Rhizobium pusense*		
13SE-122	*Streptomyces chiangmaiensis*		
13SE-125	*Pseudomonas geniculata*	X	
13SE-126	*Pseudomonas geniculata*	X	
13SE-206A	*Burkholderia contaminans*	X	X
13SE-207A	*Burkholderia contaminans*	X	X
13SE-220	*Burkholderia cenocepacia*	X	X
13SE-221	*Burkholderia contaminans*	X	X
13SE-264	*Pseudomonas brassicacearum*	X	X
13SE-333	*Burkholderia ambifaria*	X	X
13SE-334	*Burkholderia cenocepacia*	X	X
13SE-353	*Burkholderia cenocepacia*	X	X
13SE-373	*Burkholderia ambifaria*	X	X
13SE-393	*Burkholderia cenocepacia*	X	X
13SE-397	*Burkholderia ambifaria*	X	X
13SE-397	*Burkholderia ambifaria*	X	X
13SE-402	*Burkholderia cenocepacia*	X	X
13SE-406	*Burkholderia ambifaria*	X	X
13SE-407	*Burkholderia contaminans*	X	X
13SE-408	*Burkholderia ambifaria*	X	X
13SE-409	*Burkholderia ambifaria*	X	X

Fungal quantified assays are accomplished by placing an agar disc (5mm diameter) from a culture of the fungal isolate onto a PDA plate. A disc from a culture of *M. phaseolina* 151 is placed at a distance of 4cm on the same plate. The plates are incubated until growth is sufficient to determine whether there is activity against *M. phaseolina* 151. While some of the fungal isolates produce a clear zone indicative of diffusable agents that inhibit *M. phaseolina* 151 (Figure 7), others appear to grow over the *M. phaseolina* 151. We believe that the latter may be exhibiting mycoparasitism (Barnett and Lilly, 1958) and are working to develop an alternative laboratory assay that will allow efficient detection of this type of antifungal activity. As with the bacterial isolates, we are now screening all fungal isolates and will then complete the PCR identification in large batches of reactions. Representative data for fungal isolates exhibiting inhibition of *M. phaseolina* 151 and sequenced to date are presented in Table 3.

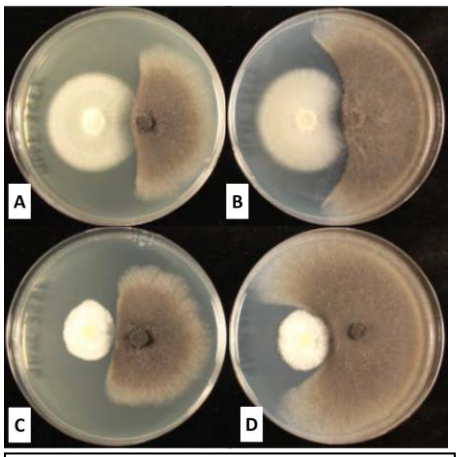

Figure 3. Inhibition of endophytic fungi isolated from asymptomatic soybean plants to *Macrophomina phaseolina* 151. An agar disc containing the isolate 151 and an agar disc (5 mm in diameter) with the tested fungus were inoculated on the potato dextrose agar (PDA) or nutrient broth yeast (NBY) agar plates. The inoculated plates were incubated for 7 days. A: 13SE-199B on NBY; B: 13SE-199B on BNY; C: Fungal isolate 13SE-204B on NBY; and D: Fungal isolate 13SE-204B on PDA.

Upon completion of all plate bioassays and identification of those bacterial and fungal isolates most active against *M. phaseolina* 151, some of the isolates will be selected for use in greenhouse studies. Isolates identified as species that are known to be human, plant or animal pathogens may not be appropriate for use as biocontrol agents. If there are known virulence factors that impact consideration of whether a species may be used in biocontrol we will analyze the strains isolated in this study for those factors.

TABLE 3. Fungal isolates displaying antifungal activity against *M. phaseolina* 151.

Isolate	Identity	
	ITS (ITS1f / ITS4)[1]	**TEF-1α[2]**
13SE-163B	*Fusarium solani*	in process
13SE-192B	*F. solani*	*F. solani* complex 3+4-y
13SE-193B	*Fusarium* sp.	*Fusarium proliferatum*
13SE-199B	*F. solani*	*F. solani*
13SE-201B	F*usarium* sp.	in process
13SE-204B	*Bionectria ochroleuca*	
13SE-206B	*Clonostachys* sp. / *B. ochroleuca*	
13SE-207B	*Clonostachys* sp. / *B. ochroleuca*	
13SE-218	*Clonostachys* sp. / *B. ochroleuca*	
13SE-225	*Neosartorya hiratsukae*	
13SE-250	*Cladospporium cladosporioides*	
13SE-285	*F. solani*	*F. solani* complex 3+4-fff
13SE-288	*F. solani*	*F. solani* complex 3+4-fff
13SE-289	*Clonostachys* sp. / *B. ochroleuca*	
13SE-295	*Plectosphaerella cucumerina*	
13SE-299	*Clonostachys* sp. / *B. ochroleuca*	
13SE-300	*Clonostachys* sp. / *B. ochroleuca*	
13SE-303	*Clonostachys* sp. / *B. ochroleuca*	
13SE-306	*B. ochroleuca*	
13SE-307	*Clonostachys* sp. / *B. ochroleuca*	
13SE-308	*Clonostachys* sp. / *B. ochroleuca*	
13SE-310	uncultured fungus / *Tremellales*	

[1]ITS identities based on BLAST results from NCBI GenBank database.
[2]TEF-1α identities from Fusarium-ID database.

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IMPACTS AND BENEFITS TO MISSISSIPPI SOYBEAN PRODUCERS

This work will lead to a better understanding of the endophytic bacterial and fungal communities present in soybean plants. The specific communities of these microbes may impact the development of charcoal rot disease. It may be possible use some of these endophytic bacteria or fungi to help the plants better resist *Macrophomina phaseolina* and/or charcoal rot disease development.

END PRODUCTS — COMPLETED OR FORTHCOMING

There have been no outputs from this project to date. We anticipate publication of three papers in referred scientific journals with one published within the next year.