**Annual Report**

**MSPB Grant #04-2024**

**Evaluation of Endophytic Bacteria in Suppression of Charcoal Rot Disease of Soybean**

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**Background and Objectives**

The total reduction in soybean yields due to charcoal rot disease caused by the fungus *Macrophomina phaseolina* in the United States was 20.8 million bushels in 2015. There are no cost-effective fungicides available for disease control and genetic resistance is not available in soybean cultivars (Tom Allen, personal communication). Endophytic bacteria are present in every plant studied, where they colonize the internal tissues of their host plants. Some endophytic bacteria (ex. *Pseudomonas* spp.) have been widely used for plant disease management. The effects of endophytes on plants can be direct or indirect; direct plant growth-promoting activities can include nitrogen fixation and increased photosynthetic potential, biosynthesis of siderophores to promote absorption of iron, biosynthesis of plant growth regulators, biosynthesis of stress defense signals, and solubilization of minerals for plant absorption.

Endophytic bacterial isolates with significant antimicrobial activities were obtained from the soybean plants collected from the charcoal rot disease patches in Mississippi. With the previous funding from the Mississippi Soybean Promotion Board and MAFES SRI, we conducted greenhouse trials for two years to evaluate the protection efficacy of some bacterial strains against the charcoal rot pathogen. The two endophytic strains of bacteria isolated from soybean plants were chosen for greenhouse testing based on results from previous in vitro bioassays. The preliminary data suggest that some bacteria (ex. MS455) promote soybean growth although they were not significantly different. Similarly, disease severity treated in the pots drenched with bacteria was reduced as compared with buffer control although there were no statistical differences. StrainMS455 shows significant antifungal activities against the charcoal rot pathogen and aflatoxin-producing fungi *Aspergillus* spp. With additional funding from MAFES and the National Corn Grower Association, the genomes of the strains MS455 and MS389 have been sequenced and evaluated in the reduction of aflatoxin in corn (Jia, et al. 2021 and Jia et al. 2022). Greenhouse and field trials demonstrate MS455 has great potential as a biocontrol agent in plant disease management against *Aspergillus flavus*, the aflatoxin-producing fungus. Genomic analysis reveals that strain MS455 genome harbors a few gene clusters, which is a homolog to the *ocf* gene cluster of the occidiofungin production of *B. contaminans* MS14.

The specific objectives for this project are 1) to investigate the effects of inoculation methods of the representative bacteria on disease development and soybean growth and 2) to characterize genes associated with antifungal activity; 3) to elucidate chemical structures of the antifungal compounds.

**Report of Progress Results/Activity**

**Objective 1: Investigate the effects of inoculation methods of endophytic bacteria into seeds on disease development and soybean growth**

* 1. **Greenhouse trials**

The following activities under this objective were conducted as proposed. Totally 50 1-Gallon pots were filled with a mixture of soil. Superior legume rhizobia inoculum was inoculated. The fungal pathogen *Macrophomina phaseolina* MP151 and the endophytic bacteria were applied into the pots and plants were maintained as planned (Table 1; Fig. 1). The soybean plants were measured twice (Aug. 5 and Sept. 6, 2024). The trails were terminated on Oct. 23, 20024.

**Plant growth observed on Aug. 5:** An ANOVA analysis was performed which indicated that there are no significant statistical differences between the treatment groups. We have observed the locations of plants affect soybean growth significantly in the greenhouse. We tried to create a drought condition to promote development of charcoal rot. Some noteworthy findings were still observed. The results indicate that the bacteria have some positive effect on the growth of the plants although the treatments are not significantly different.

**Plant growth observed on Sept. 6:** An ANOVA analysis confirmed that the treatments are not significantly different from each other. The data are shown in Fig. 2. These findings support the conclusion that the inoculation method influences disease development and plant growth, supporting the original hypothesis.

**Disease rating results on Oct. 23.** Disease development was evaluated using two different methods. Disease rating was based on the scale published by Mengistu et al. 2007. Percent height of internal stem discoloration (PHSD) was calculated according to the height of internal vascular discoloration from ground level and divided by the total stem height (Da Silva et al. 2019). An ANOVA analysis was performed on disease rating and PHSD. Disease ratings of Control 1 and Treatment 3 were found to be statistically different in addition to Treatment 3 and Treatment 1. The only pair showing statistical difference in PHSD were Treatments 3 and 1. These findings reinforce the hypothesis that different inoculation methods and concentrations of plant growth-promoting bacteria influence disease development. The detailed data are shown in the Table 2 and Fig. 3,

* 1. **Trials to introduce MS455 into soybean seeds**

Five pots of soybean plants were used for introduction of the bacteria into seeds via flowers. As scheduled, the wild-type bacterial strain MS455 were sprayed on Aug. 30, 2024, onto soybean flowers. Phosphate buffer was used as a control. The soybean seeds were harvested on Oct. 23, 2024, for examination of presence of MS455. The wild-type strain MS455 possesses a natural antibiotic resistance to kanamycin. Because we had not labeled MS455 using the GFP fluorescence gene that time, we had to rely on the resistance marker for examination of presence of MS455 in soybean seeds. All the seeds obtained from the inoculated soybean plants (~110 pods) and phosphate buffer control (~100 pods) were surfaced sterilized, ground and used for bacterial isolation on NBY agar plates supplemented with 50 mg/L of kanamycin. No single MS455 colonies were received from both control and inoculated soybean seeds based on sequence analysis.

After the failure to create MS455-GFP using plasmid pUTminiTn5-GFP, two other plasmids were used for label MS455. We finally obtained MS455-GFP using the plasmid pSL-1-GFP, which were confirmed by using plasmid extraction and digestion, and further by observation of the GFP expression (Fig. 4). Stability of the GFP in the MS455 cells is under investigation.

**Objective 2: To characterize the genes associated with antifungal activity.**

The whole genome of the bacterial strain MS455 has been downloaded from GenBank. Analyses of the whole genome for search more antimicrobial activities-related genes were conducted by using various software, such as antiSMASH (Blin, 2023). As expected, the gene cluster responsible for production of the antifungal compound occidiofungin variant was identified (Table 3), which shares 94% identity to that of the bacterial strain MS14 (Gu et al. 2009). Additional genes, involved in biosynthesis of the antimicrobial compounds Orinbactin (Deng et al. 2017), Terpene (Mahizan et al. 2019) and Pyrrolnitrin (Arima et al. 1964) were found from the MS455 genome (Table 3). Interestingly, one genetic locus is present in the MS455 genome, which shares 14% similar to the gene that is involved in biosynthesis of endopyrrole. Endopyrrole may be essential for fungal–bacterial symbiosis between organisms (Niehs et al. 2019). MS455 is an endophytic bacterium in soybean. It would be extremely interesting to verify its role in the endophytic phenomenon.

Approximately 20 bacterial isolates, which showed antimicrobial activity against plant pathogens have been isolated from rhizosphere of soybean plants collected from suppressive soils of Mississippi. Preliminary identification using the 16S rDNA sequencing demonstrated majority of them belongs to *Bacillus*, *Pseudomonas*, *Burkholderia* and *Streptomyces*. Further analyses are under way to determine their taxonomic positions. The isolate EA59 (*Bacillus siamensis*) and strain MS455 were used to evaluate their efficacy of growth promotion to *Camelina sativa* in growth chamber. The results showed both bacteria promoted growth of the plant with increased leaf number and fresh weight. In addition, a few isolates (EA56, EA65 and EA67) were obtained, showing impressive antimicrobial activities. The 16S rDNA sequence analysis indicates some belong to *Streptomyces* spp.

**Objective 3: To elucidate chemical structures of the antifungal compounds.**

We are collaborating with Dr. James Smith at Texas A&M University to elucidate the chemical structure of the MS455 antifungal compound. Extraction of antifungal compounds were conducted as described previously (Gu et al. 2009). As shown in Fig. 5, antifungal compounds produced by the strain MS455 may be a complex of a few compounds, which include the HPLC fractions 6, 7, 9. No 6 shows the strongest activity against the indicator fungus *Saccharomyces cerevisiae*. Currently, more purified antifungals are being prepared for further analysis of chemicals structure using TOCSY, NOESY, ROESY as described previously (Lu et al. 2009). The work on MS398 was collaborated with the former MSU professor Xin Cui. Unexpectedly, Dr. Cui moved to Dallas, Texas and the collaboration was terminated in October 2024.

**Impacts and Benefits to Mississippi Soybean Producers**

This research has discovered more bacterial isolates that possess antifungal activities. In vitro assays, these isolates showed significant inhibition to growth of the charcoal rot pathogen *Macrophomina phaseolina*. Strains MS455 and MS389 have showed great potentials as biological control bacteria on soybean. These bacteria are very important resources for the development of biologically based management approaches to soybean disease. More extensive studies on genetics of antifungal production of the two strains have provided insights to understanding regulations of the antifungal compounds. Effects of three of them on development in greenhouse will be further investigated. The trial will provide important data for possible use of the isolates. Elucidation of chemical structure of the antifungals is extremely useful for development of biopesticides. Collectively, the expected outcomes of the research will provide critical insights to development of products to help soybean plants better resist infection of *Macrophomina phaseolina* and prevent charcoal rot disease development.

**End Products–Completed**

**Peer-reviewed journal publication:**

Willis, Emma, Wes Phillips, Daniel Jeffers, and Shi-En Lu. "Complete genome resource for *Pseudomonas* sp. strain WP18 isolated from a disease suppressive soil." PhytoFrontiers (2025): doi.org/10.1094/PHYTOFR-07-24-0084-A. This paper approved by Dr. Jason Krutz for publication is part of the MS thesis of Ms. Emma Willis, who was granted the MS degree in May

2025.

**Presentation:**

Khan, Bailey, Ehtasham Ali, Lindsey Robinson, and Shi-En Lu. “Identification and Characterization of Antimicrobial Bacterial Isolates from the Mississippi Delta”. Mississippi State University's Spring 2025 Undergraduate Research Symposium on April 9-10, 2025, in the Colvard Student Union.

**Graphics/Tables**

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| **Table 1. Treatments and Controls in 2024** | |
| Controls | CK1: MP151 only |
| CK2: MS455 only (OD420= 0.5) |
| CK3: MS389 only (OD420= 0.5) |
| Treatments | T1: MP151 + MS455 drenching at seeding (OD420= 0.5) |
| T2: MP151 + MS455 drenching at seeding & R1 stage (OD420= 0.5) |
| T3: MP151 + MS389 drenching at seeding and R1 stage (OD420= 0.5) |
| T4: MP151 + MS455 priming at seeding (OD420= 0.5) |
| T5: MP151 + MS455 priming at seeding (OD420 = 1.0) |
| T6: MP151 + MS389 priming at seeding (OD420= 1.0) |

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| **Fig. 1.** Greenhouse trial of endophytic bacteria to suppress charcoal rot disease of soybean. | |

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| **Fig. 2.** Growth and development of soybean plants treated with *Burkholderia* spp. MS455 and MS389 (Sept. 6, 2024). Treatments (T1-6) and controls (CK1-3) are described in detail in Table 1. |



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| Several pieces of wood  Description automatically generated |
| **Fig. 3.** Split lower stem and root sections showing disease severity of the charcoal rot caused by Macrophomina phaseolina. soybean plants (Oct. 23, 2024). Treatments (T1-6) and controls (CK1-3) are described in detail in the proposal narrative. |

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| A close-up of a petri dish  AI-generated content may be incorrect. | **Fig. 4.** The endophytic bacterium MS455 expressing green fluorescence protein observed under ultra-violent light at 395 nm. The MS455 cells were electronically transformed with the plasmid pSL-1-GFP. After transformation, the cells were plated on culture medium supplemented with kanamycin (225 µg/mL). The green colonies express the GFP gene. These small and non-green colonies are GFP negative. |

**Table 3.** Antimicrobial genes identified from the MS455 genome based on the antiSMASH analysis

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| **Chromosome** | **Location** | **Product** | **Similarity** |
| 1 | 1,721,277-1,786,337 | Orinbactin | 100% |
| 2 | 1,294,576-1,315,640 | Terpene | 50% |
| 2 | 1,517,180-1,569,223 | Pyrrolnitrin | 100% |
| 2 | 2,132,078-2,153,875 | Endopyrrole | 14% |
| 3 | 338,339-423,668 | Occidiofungin | 94% |

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| **Fig. 5.** Antifungal compounds produced by *Burkholderia* sp. strain MS455. Left: Purification of the MS455 antifungal compounds using high-performance liquid chromatography (HPLC). Right: HPLC fractions (6, 7, 9, and 10) against the fungus *Saccharomyces cerevisiae*. |