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Project Title: Characterization of Antifungal Activity of Endophytic Bacteria Associated with Soybean – Charcoal Rot Disease System

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BACKGROUND AND OBJECTIVES

Charcoal rot disease, caused by the fungus *Macrophomina phaseolina*, accounts for an estimated loss of 1.48% of soybean yield in the Southern states. This disease is particularly problematic due to the lack of fungicides capable of providing effective disease control, and lack of genetic resistance in cultivars.

Endophytic bacteria occur in all plants and we have demonstrated some differences in endophyte communities of diseased and asymptomatic plants growing adjacent to one another in diseased patches of soybean fields. Our previous MSPB-funded investigations resulted in the identification of a number of bacterial isolates that show antifungal activity against *M. phaseolina*. Preliminary studies indicate that these endophytic bacteria may play a key role in helping plants resist infection by the charcoal rot pathogen and/or inhibit disease development in soybean fields.

The long-range goals of the research program are to develop biologically-based approaches to plant disease management. The specific objectives for this project are 1) investigation of the effects of inoculation of the representative bacteria on disease development and soybean growth, and 2) characterization of the genes associated with antifungal activity via transposon-mediated muutagenesis. Expected results will provide a solid basis for development of charcoal rot disease management for the Mississippi soybean industry.

REPORT OF PROGRESS/ACTIVITY

Objective 1: Investigation of the effects of inoculation of bacteria and fungi on disease development and soybean growth.

***In vitro* assays and Preliminary identification.** Quantitative *in vitro* evaluation of direct interactions between more than 300 endophytic bacterial isolates and the fungal pathogen *M. phaseolina* was conducted. As the pool of most promising isolates was narrowed, bacterial isolates were grouped based on colony morphology and representative members of each group were subjected to PCR amplification and sequencing of the 16S rDNA and other regions of the genome. To date, over 50 isolates have been tested in this manner. These efforts focused on five strains that have excellent in vitro activity against *M. phaseolina*. Of these, four isolates were determined to belong to the genus *Burkholderia* and one belongs to the genus *Paenibacillus*. Three isolates, two *Burkholderia* species and one *Paenibacillus* species, were selected for inclusion in greenhouse *in vivo* testing.

Preliminary greenhouse assays. Asgrow 4651, the soybean variety from which the endophytic bacterial strains were isolated, is no longer available. Consequently, two soybean varieties were used to assure that at least one of them is susceptible to *M. phaseolina* strain Mp151 that we used for *in vitro* work.

Pioneer 46A16R and Viking 2265 were planted in one-gallon pots containing a mixture of commercial potting soil and sand which was steam-sterilized prior to use. Two methods of inoculation with the fungal pathogen were tested: (1) incorporation of fungal inoculum into the soil prior to planting; and (2) inoculation of the fungal pathogen into wounded stems of young plants grown in sterile soil mixture.

Plants were destructively sampled twice—half of them at physiological maturity (pod set) and half at natural senescence. Four 1.5-cm-long sections of stem and root, measured at consecutive intervals out from the soil line, were cut, surface-sterilized, split longitudinally, and placed onto plates of PDA supplemented with 100µg/mL ampicillin. The remaining stem of each plant harvested at senescence was stored at -80°C for future work.

Although only two plants, both of them Viking 2265, displayed classic charcoal rot disease symptoms, *M. phaseolina* was isolated from at least one stem and one root section from most plants. No *M. phaseolina* was isolated from one plant. *M. phaseolina* was isolated from fewer stem and root sections of plants inoculated at wounded stems as compared to those planted into infested soil.

Future studies will use only Pioneer 46A16R as the soybean variety and infested soil as the inoculation method. The lack of classic disease symptoms in this test indicates that future greenhouse studies will require metrics other than disease symptomology to evaluate efficacy of the bacterial isolates for *in vivo* control of *M. phaseolina*. To that end, we will use the stored stem tissues from the preliminary test to establish our methods for DNA extraction and quantitative real-time PCR (qPCR) using primers specific for *M. phaseolina*. We believe that qPCR combined with biomass measurements will yield satisfactory metrics of biocontrol efficacy.

Greenhouse assays. The large-scale greenhouse study was set up in June 2018. Prior to setting up the test, a drip irrigation system fed from overhead lines was constructed in the greenhouse. This system will prevent overspray and splash when plants are watered, providing the greatest assurance that cross-contamination does not occur between the various treatments.

The soil/sand mixture tested in the preliminary assays was prepared and steam-sterilized prior to use. Treatments are as described in Table 1. To prepare inoculum of *M. phaseolina* Mp151, 40 flasks of corn meal sand medium (100g sand, 3g corn meal, 20mL distilled water per flask) were prepared and autoclaved. Four 3-mm plugs from plates of Mp151 on PDA were placed into each flask. Flasks were incubated in the dark at 30°C for 10 days and shaken well by hand at 3-day intervals, at which time the contents of all flasks were combined and mixed. Each pot containing Mp151 was prepared by hand-mixing 20mL of this inoculum into the sterile soil.

Pots for treatments not containing Mp151 were filled with sterile soil into which was mixed 20 mL of sterile corn meal sand medium. Bacterial inoculum of the three selected endophytic isolates was prepared by inoculating the bacteria into flasks of NBY liquid medium that were then incubated 18 hours at 28°C with 220 rpm shaking. Bacteria were extracted from the medium by centrifugation and resuspended in sterile 0.01M PBS, pH 7.4.

Bacteria were inoculated into the seed furrow at the rate of 10^9 cfu in 50 mL PBS per pot. Three *Rhizobium*-inoculated Pioneer 46A16R soybean seeds were placed in the furrow, and the seeds were covered with soil.

Pots that did not receive bacteria were treated similarly by pouring 50 mL of sterile PBS into the furrow. Four replicates with three pots for each of the eight treatments were prepared and pots were distributed on greenhouse benches in a randomized complete block design. One 1 gpm pressure-compensating drip emitter

was placed into each pot for irrigation. When the plants reach the V1 growth stage, they will be thinned to one plant per pot. Plants will be destructively sampled at natural senescence. Data will include the effects of these bacterial strains on disease development, pathogen population, plant growth, and disease resistance in Pioneer 46A16R soybeans.

Objective 2: Characterize the genes associated with antifungal activity production.

Characterization of genetic materials required for production of antifungal activity is critical for development of biological-based disease management approaches. As described previously, we have isolated more than 50 endophytic bacterial isolates from soybean plants. Our efforts for characterization of genetic elements for production of antifungal activities have been centered on two isolates (A and B) which show significant antifungal activities, and their effects on charcoal rot disease development are under investigation (Objective 1)

In brief, competent cells of each isolate were prepared for introduction of transposon by electroporation. A mixture of competent cells with transposome (Lucigen, Madison, WI) was electroporated and transformants were plated on agar plates supplemented with kanamycin (Km). The resulting colonies growing on Km plates were picked up and phenotypic screening was conducted using a standard bioassay protocol. We have generated a few mutants of the isolates A and B.

Preliminary analysis indicated that the two isolates belong to the genus *Burkholderia* but with different phenotypes including colony features. A few mutants of the three isolates were obtained and the mutants either completely or partially lost their activity against a few pathogenic fungi including *M. phaseolina* (Figs. 1 and 3). The partial sequences of the genes disrupted in the mutants do not share significant similarity with the *ocf* genes of *B. contaminans* MS14. More interestingly, the mutant A-36 showed no antifungal activity against charcoal rot pathogen (*M. phaseolina*), but exhibited a different spectrum of antifungal activities as compared the known antifungal compounds produced by the bacteria *Burkholderia*, including occidiofungin

Preliminary genetic analysis also indicates that the antifungal compound produced by isolate A is different from the known antifungals produced by *Burkholderia*. A 57-kb gene cluster has been sequenced, which includes the genes associated with nonribosomal biosynthesis, transcriptional regulation, modification, and secretion (Fig. 4). In collaboration with Dr. Nicholas Fitzkee, Department of Chemistry, we plan to elucidate the chemical structure of the antifungal peptide. Production of the compound in liquid culture media is under investigation for purification. Similarly, isolate B was mutated for gene identification. The mutant B-44 did not produce visible antifungal activity against indicator fungus *G. candidum* (Fig. 2). Preliminary studies showed one gene that encodes a LysR family regulator was disrupted in the mutant. Further genetic characterization of the isolate is underway.

IMPACTS AND BENEFITS

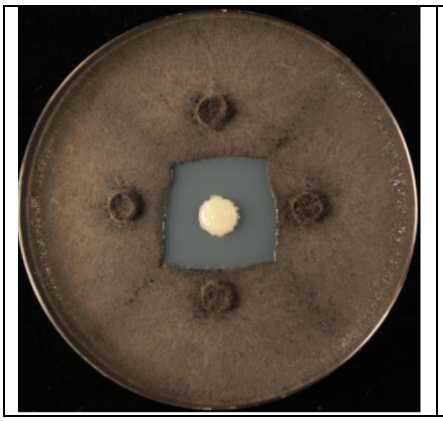
This research has discovered more than 50 bacterial isolates that possess antifungal activities. Using in vitro assays, these isolates showed significant inhibition to growth of the charcoal rot pathogen *Macrophomina phaseolina*. These bacteria are a very important resource for development of biologically-based management approaches of the soybean disease. Effects of three of them on disease development in the greenhouse are under evaluation.

The trial will provide important data for possible use of the isolates. The genes responsible for production of antifungal activity could be used to develop genetically modified organisms for disease control. Elucidation of the 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 OR FORTHCOMING

There have been no outputs from this project to date. We anticipate publication of two papers in refereed scientific journals with one published within the next year and development of microbial package for charcoal rot disease management.

Table 1. Greenhouse assay treatments.		
Treatment Name	**Fungal Inoculum**	**Bacterial Inoculum**
Negative Control	20mL sterile cornmeal sand medium	50mL sterile buffer
Mp151 Control	20mL Mp151 inoculum	50mL sterile buffer
Isolate A Control	20mL sterile cornmeal sand medium	10^9 cfu Isolate A in 50mL buffer
Isolate B Control	20mL sterile cornmeal sand medium	10^9 cfu Isolate B in 50mL buffer
Isolate C Control	20mL sterile cornmeal sand medium	10^9 cfu Isolate C in 50mL buffer
Isolate A + Mp151	20mL Mp151 inoculum	10^9 cfu Isolate A in 50mL buffer
Isolate B + Mp151	20mL Mp151 inoculum	10^9 cfu Isolate B in 50mL buffer
Isolate C + Mp151	20mL Mp151 inoculum	10^9 cfu Isolate C in 50mL buffer



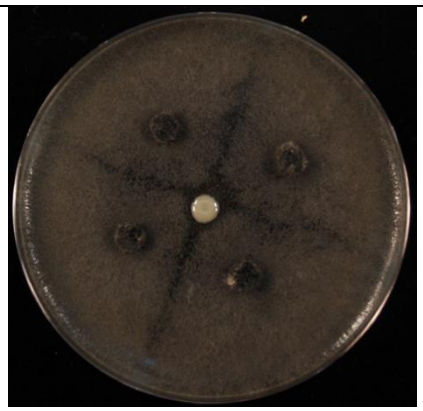


Fig. 1. Quantified plate bioassays of an endophytic bacterial strain (Isolate A; left) and its mutant (right) against charcoal rot pathogen (*M. phaseolina*). Presence of transposon was verified by PCR analysis.

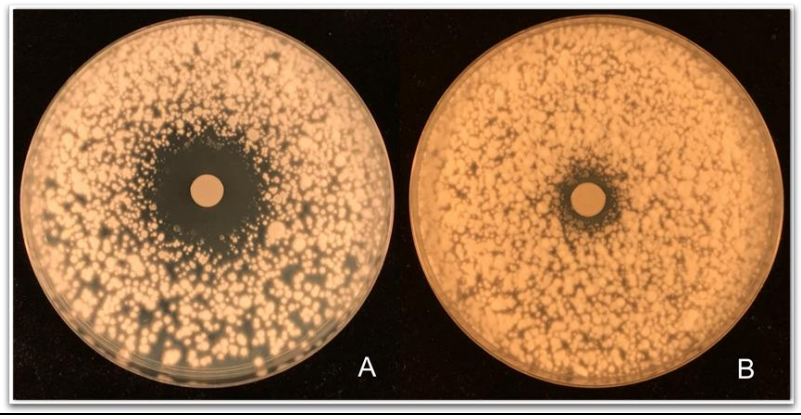


Fig. 2 Antifungal activity *Burkholderia* sp. isolate B against *Geotrichum candidum* was lost in mutant B-44. A: isolate B, B: mutant B-44. Presence of transposon was verified by PCR analysis.

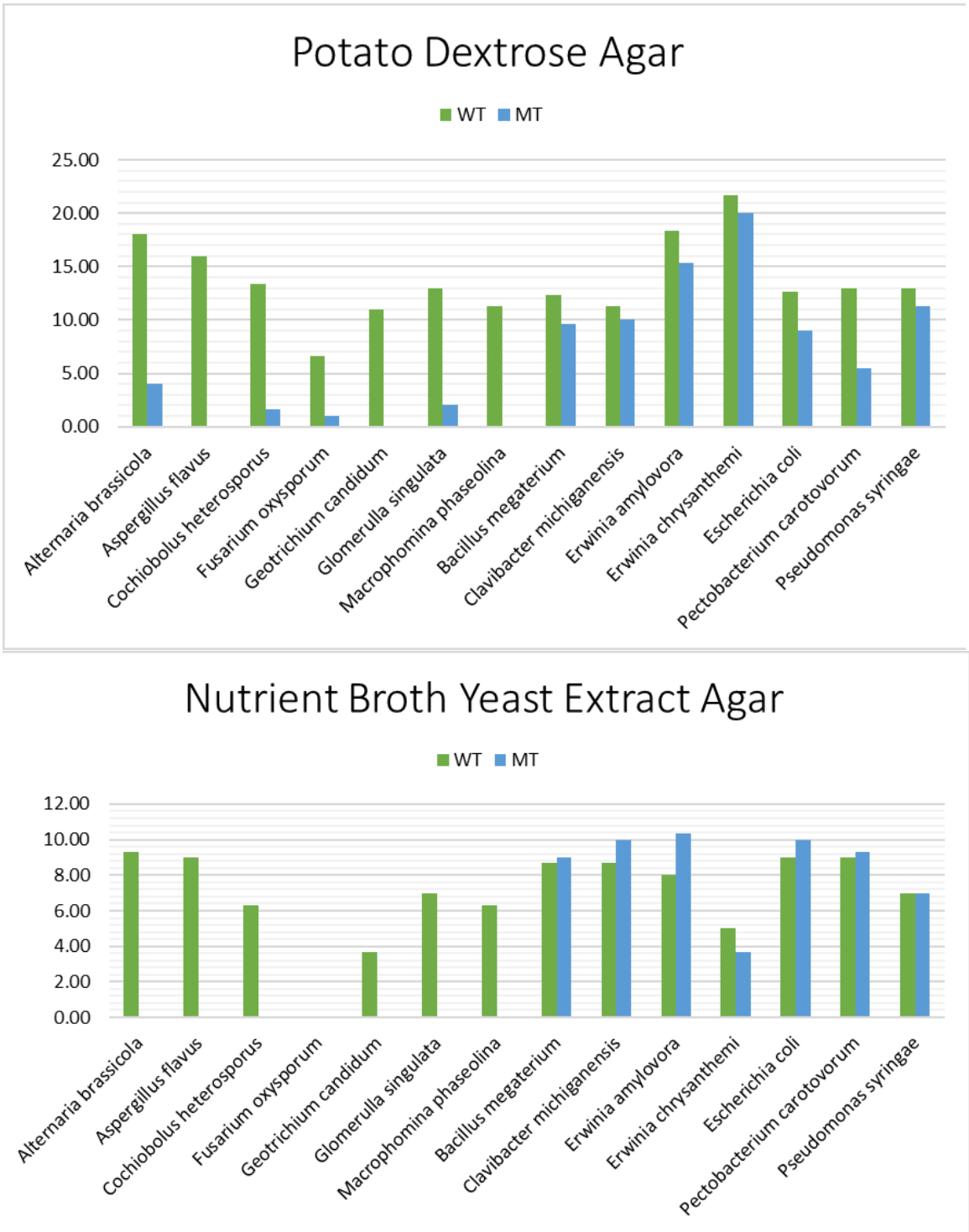


Fig. 3. The plate bioassays were replicated three times; bars represent standard deviations. Antifungal activities of the wild type strain and it mutant were compared against different fungi using the standard bioassay protocol on potato dextrose agar (upper) and nutrient broth yeast extract agar (bottom) plates.

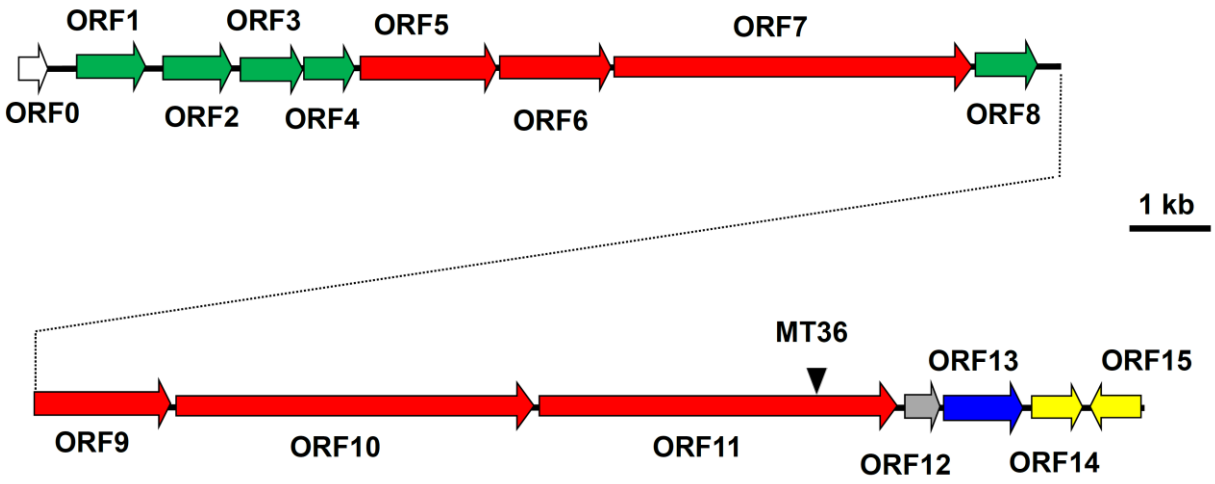


Fig. 4. The 57-kb gene cluster required for antifungal activity of endophytic bacteria *Burkholderia* sp. isolate A. MT36 is a mutant of isolate A and the triangle is the location of transposon.