MISSISSIPPI SOYBEAN PROMOTION BOARD

CHARACTERIZE ROOT MICROBIAL COMMUNITIES WITH ANTI-FUNGAL ACTIVITIES IN SOYBEAN
PROJECT 53-2022
ANNUAL REPORT

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RATIONALE/JUSTIFICATION FOR RESEARCH

Plant roots associate with a microbial community that is distinct from the microbes present in surrounding soil. The microbes colonize the rhizosphere (immediately surrounding the plant's roots) and the superficial root tissues (endophytic compartment). The root microbiota comprises a wide diversity of microorganisms, and it can benefit plant health or have detrimental effects; shifting this balance towards beneficial plant-microbiota interactions is of high agronomic interest. Microbes may provide the plant with nutrients that accelerate plant growth and development, suppress soil pathogens, or help plants withstand environmental stress. The advantages of exploiting microbiota are evident and include consumers' and farmers' safety and sustainable practices that preserve the environment and protect biodiversity.

Taproot decline (TRD), caused by *Xylaria* sp., is recognized as a critical soil-borne soybean disease. Previous MSPB studies by Dr. Maria Tomaso-Peterson demonstrated TRD is widespread throughout MS soybean production, and affected soybeans were identified in 73 counties. Through the support of MSPB, we are developing a better understanding of TRD and the causal agent *Xylaria* sp. This pathogen overwinters as stroma on crop residue across successive growing seasons and colonizes the roots of corn, cotton, rice, sorghum, and wheat in greenhouse studies. We demonstrated variable degrees of virulence among *Xylaria* sp. isolates, which may be due to microbes' influence within the soybean plants' rhizosphere. Field observations show dead soybean seedlings resulting from TRD at the V4–V5 growth stage; however, most symptomatic soybeans observed in production fields are in growth stages greater than R3.

Soil microbiome investigations have been strongly promoted over the past ten years. Microbiome studies have provided insights into the composition of disease-suppressing soils. For example, soil suppressing wilt disease caused by Ralstonia solanacearum contains a higher abundance of *Proteobacteria* and *Acidobacteria* than soils with disease symptoms. Species with anti-fungal activity include non-pathogenic strains of *Fusarium oxysporum* and bacteria in the genera *Pseudomonas* and *Bacillu*s. Thus, the evaluation of root microbiomes for crop growth promotion and disease resistance presents unique

opportunities. We aim to understand the diversity of rhizobiome microorganisms that cause specific phenotypes in soybeans, test rhizobiome isolates for beneficial activities, and utilize this knowledge to establish methods for improved growth and biological control of soil pathogens.

Primary goals of the project:

1: Identify rhizobiome microorganisms with inhibitory activity on *Xylaria* growth

2: Characterize the rhizobiome communities of the healthy and diseased soybean to quantify the impact of *Xylaria* infection on the composition and structure of soybean rhizobiome.

3: Evaluate the biocontrol activity of rhizobiome microorganisms against soybean taproot decline in the field.

Activities and significant results:

1. **Phylogenetic analysis of bacterial endophyte library:** We integrated all experimental and phylogenetic analysis results to generate a list of root endophyte library (REL) isolates, their species identification and an estimated of X. necrophora inhibitory coefficient (**Table 1**). The group of bacteria with anti-TRD activity identified includes Is-4, Is-7, Is-9, Is-10, Is-15, Is-20, Is-21, Is-40, Is62, Is-65, Is-66, Is-71, Is-72, Is-78, Is-84, Is-89, Is-111, Is-113, and Is-148.
2. **In planta testing of beneficial isolates using soil collected from agricultural fields.** In this quarter, we repeated in planta assays of isolates that demonstrated strong anti-Xylaria and anti-TRD activity in previous experiments (isolates 9, 10, 15, 21, 62, and 71) to confirm their anti-TRD capabilities (Fig. 1). All isolates protected soybeans effectively from TRD. All bacterial-treated soybeans with Xylaria inoculation exhibited interveinal chlorosis in the lower canopy, but all remained alive and appeared significantly healthier than control soybeans with Xylaria (**Fig. 1A, B**). Soybeans treated with Is-62 (**Fig. 1.C**) and Is-71 (**Fig. 1.D**) displayed an improvement in overall development, demonstrating their capacity to promote plant growth.
3. **Design and implemented a field trial to test selected bacterial isolates for inhibitory activity against *Xylaria necrophora*.** Trial map and position of each sample/treatment in the randomized complete block is shown in **Figure 2**. Field trial started on June 7, 2022 and described in the previous quarterly report. During the field trial, several plant measurements were collected to assess TRD disease phenotype. The first data was collected on 06/29/2022. Harvest data was collected at the end of the season. Initial data, field trial image data (**Figure 3**) and harvest data were analyzed using statistical methodology to assess bacterial isolates effect.
4. ***In vitro* Bacterial-Fungal Cocultivation Assays with Synthetic Communities:** For a co-cultivation experiment using synthetic communities (syncoms), isolates with moderate to high anti-*X. necrophora* and anti-TRD characteristics were selected. This experiment was intended to investigate the interaction and efficacy of various combinations of bacterial isolates against *X. necrophora*. Isolates 9, 10, 20, 62, and 72 were used to construct 26 syncoms, including 1 syncom of five isolates, 5 syncoms of four isolates, 10 syncoms of three isolates, and 10 combinations of two isolates. Cocultivation trials of *X. necrophora* with Is-9, Is-10, Is-20, Is-62, and Is-72 were conducted as five distinct controlled variables, in addition to a control trial containing no bacteria. Seven syncoms inhibited X. necrophora growth by less than 15%, nine syncoms inhibited X. necrophora growth by 15% to 25%, and ten syncoms had the most substantial fungal inhibition effects of 25% and higher (**Figure 4**).
5. **Developed a predictive understanding of TRD development from metagenomics data sequenced by COSMOSID.** We developed a statistical framework for analyzing the TRD

development from metagenomics data sequenced by COSMOSID. We performed correlation and network analysis between *Xylaria* and other bacterial and fungal strains (**Figure 5**). We generated a predictive understanding of how bacterial strains impact *Xylaria* accumulation over the disease progression (**Figure 6**).

Key research outcomes:

1. Identification of bacterial isolates composition and estimation of X. necrophora inhibitory coefficient for the endophyte library.

2. Third *in planta* testing of beneficial isolates using *soil collected from agricultural fields*: we confirmed strong anti-Xylaria and anti-TRD activity of isolates selected in previous screens (isolates 9, 10, 15, 21, 62, and 71) before the field trials.

3. Results of initial field trails: field trials were planted on June 7, 2022, to an Asgrow brand soybean, AG43XO. The experimental treatments included eight bacterial isolates previously observed to have activity minimizing the effect of *X. necrophora*: Is-9, Is-10, Is-15, Is-20, Is-21, Is-62, Is-71, Is-72.

4. Results of *in vitro* bacterial-fungal co-cultivation assays with synthetic communities. Several combinations of isolates (Is-9, 10, 20, 62, and 72 were used to construct 26 syncoms) were tested *in vitro*. The results are used to design next year field trials.

Opportunities for training and professional development provided by the project:

1. Training of undergraduate students in microbiology, molecular, computational biology and plant biology techniques:

   Tyrikus Hayes - helped with soybean growth, plant maintenance and tissue processing at the end of each experiment. Tyrikus was accepted into medical school (MS) and started his graduate studies in August 2022

   Joshua Mitchell- helped with organizing work on the project, optimizing assays for soybean growth, plant maintenance and tissue processing at the end of each experiment. Josh was accepted in the Graduate School at MSU (Dept. of Biochemistry) and started working under the supervision of Dr. S. Popescu

   Sean McGrath– helped with soybean growth and plant maintenance, and assisted processing soybeans and measuring dry mass at the end of the experiment.
   Fenny Patel - helped with soybean plant maintenance and tissue processing at the end of each experiment

   Stephanie Dauber – helped with analysis of plant images and metagenomic data analyses; funded through an URSP project: Computational analysis of root microbial communities with anti-fungal activities in soybean

2. Training of graduate students in metagenomics and lab techniques for biocontrol:

   Joshua Mitchell, beginning graduate student: optimizing assays for soybean growth, plant maintenance, and field trials, quantification of field trial images.

   Jasmine Uyen Weser, Master's student: microbiological and molecular lab work, plant assays, field assays; graduated in December 2022 with a Master of Science in Biochemistry

in the Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology.

Philip Berg, Ph.D. student, designed metagenomics and microbial community data analysis methods, performed soybean data analysis, supervised undergraduate students working on data analysis. Graduated in December 2022 and moved into a postdoctoral position.

3. Training of one postdoc, **Dr. Xin Ye** in microbiology, molecular and plant biology techniques

Products and dissemination of results to communities of interest:

1. Video presentation at the DREC meeting in July 2022 by the PI.
2. Journal paper published: Popescu SC, Tomaso-Peterson M, Wilkerson T, Bronzato-Badial A, Wesser U, Popescu GV. Metagenomic Analyses of the Soybean Root Mycobiome and Microbiome Reveal Signatures of the Healthy and Diseased Plants Affected by Taproot Decline. Microorganisms. 2022 Apr 21;10(5):856. doi: 10.3390/microorganisms10050856. PMID: 35630301; PMCID: PMC9143508.
3. Master's student thesis defense: Uyen "Jasmine" Wesser defended and published her thesis: "Metagenomic analysis of root-associated microbiome of healthy and Taproot Decline-affected soybeans and identification of healthy soybean root endophytes with protective activity against the causal agent, *Xylaria necrophora*". https://orcid.org/0000-0002-1536-4160
4. Introduction to Research Methods Presentation: Master's student Uyen Wesser presented her thesis experiments and PhD student (**Fig. 7A**); Josh Mitchell presented field trial analysis to high school students in their Introduction to Research Methods course at MSU (**Fig. 7B**).

Impact of the project

Scholarly Impact. Publications, oral presentations at regional conferences, training of undergraduates, Ph.D., and Master Students, and posting research results and conclusions on the MSPB website (www.mssoy.org). Creation of an entry in the National Soybean Research Database: https://www.soybeanresearchdata.com/Project.aspx?id=54983 and dissemination on website: www.SoybeanResearchInfo.com.

Economic impacts. Taproot decline (TRD) is widespread throughout MS and Southern US and cannot be controlled through classical methods. We aim to discover and deploy methods for the biological control of *X. necrophora* for enhancing soybean production by reducing TRD incidence. Our results provide evidence and proof-of-principle for the development of synthetic bacterial communities for targeted use against *Xylaria* sp.

Our overarching aim is to produce probiotic products for use in Mississippi agricultural fields to suppress disease.

Table 1. *X. necrophora* inhibition activity for identified REL species

REL nr.	Genus, Species	*X. necrophora* inhibition (%)
Is-4	*Burkholderia ambifaria* *Enterobacter hormaechei* *Enterobacter ludvigii*	35
Is-7	*Burkholderia ambifaria* *Enterobacter hormaechei* *Enterobacter ludvigii*	33
Is-8	*Enterobacter cloacae*	-12
Is-9	*Burkholderia ambifaria*	33
Is-10	*Acinetobacter calcoaceticus* *Acinetobacter oleivorans*	40
Is-11	*Acinetobacter calcoaceticus* *Acinetobacter oleivorans* *Burkholderia ambifaria*	12
Is-12	*Enterobacter ludwigii*	18
Is-13	*Enterobacter ludwigii*	8
Is-14	*Enterobacter cloacae*	15
Is-15	*Enterobacter cloacae* *Enterobacter ludvigii*	36
Is-16	*Enterobacter ludvigii*	13
Is-17	*Enterobacter ludwigii*	5
Is-18	*Enterobacter ludwigii*	4
Is-19	*Enterobacter cancerogenus*	7
Is-20	*Enterobacter cloacae* *Enterobacter ludvigii*	21
Is-21	*Enterobacter cloacae* *Enterobacter ludvigii*	28
Is-23	*Enterobacter cloacae*	-19
Is-31	*Pseudomonas panacis*	-18
Is-33	*Enterobacter asburiae*	-19
Is-37	*Pseudomonas moraviensis*	-12
Is-39	*Pseudomonas panacis* *Enterobacter asburiae*	14
Is-40	*Enterobacter asburiae*	29
Is-41	*Enterobacter cloacae*	18
Is-62	*Enterobacter asburiae*	46
Is-65	*Pseudomonas panacis*	44

Is-66	*Enterobacter asburiae*	19
Is-71	*Pantoea agglomerans* *Pantoea vagans*	35
Is-72	*Pantoea vagans* *Rhizobium pusense*	29
Is-77	*Enterobacter asburiae*	19
Is-78	*Enterobacter asburiae* *Enterobacter ludvigii*	31
Is-83	*Enterobacter asburiae*	24
Is-84	*Achromobacter xylosoxidans* *Enterobacter asburiae* *Enterobacter ludvigii*	37
Is-88	*Enterobacter asburiae*	22
Is-89	*Enterobacter asburiae*	28
Is-111	*Enterobacter asburiae*	46
Is-113	*Enterobacter asburiae* *Enterobacter ludwigii*	52
Is-131	*Enterobacter asburiae*	21
Is-132	*Enterobacter asburiae*	18
Is-148	*Enterobacter asburiae*	30
Is-150	*Enterobacter ludwigii* *Enterobacter hormaechei*	23
Is-151	*Enterobacter ludvigii* *Enterobacter hormaechei*	21
Is-152	*Enterobacter ludvigii*	17

Red-highlighted cells represent high inhibitory activity that inhibited the growth of *X. necrophora* by at least 25%. Pink-highlighted cells represent moderate inhibitory action limiting *X. necrophora*'s growth by 12% to 24.99%. Gray-highlighted cells represent low to no inhibition activity (<12%). Negative inhibition percentage indicated an adverse effect in which the growth of *X. necrophora* was increased by the bacteria.

Figure 1. Analysis of bacterial-treated soybeans with and without Xylaria inoculation

A. Control root

B. Control plant

C. Is-62

D. Is-71

E. Is-9 F. Is-10

G. Is-15 H. Is-21

Figure 2. Trial map and position of each sample/treatment in the randomized complete block.

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Treatment	Code	Description
1		**Untreated**
2		**Is-9**
3		**Is-10**
4		**Is-15**
5		**Is-20**
6		**Is-21**
7		**Is-62**
8		**Is-71**
9	CHK	**Is-72**

1099	2095	3092	4094
1088	2086	3089	4081
1077	2079	3083	4078
1067	2067	3066	4067
1055	2053	3051	4052
1044	2048	3044	4049
1033	2032	3035	4033
1022	2021	3027	4026
1011	2014	3018	4015

Figure 3. Images of plants from the field trial. Images were taken on 07/12/2022, approximately 1 month after planting. These images will be analyzed, and the canopy area will be quantified for all treated/untreated and inoculated/un-inoculated plants to determine the possible effect of bacterial treatments on TRD development and plant growth.

Figure 4. Bar graph displaying the percentage of inhibition by the 26 synthetic community (syncom) combinations and 5 individual isolates against *X. necrophora*.

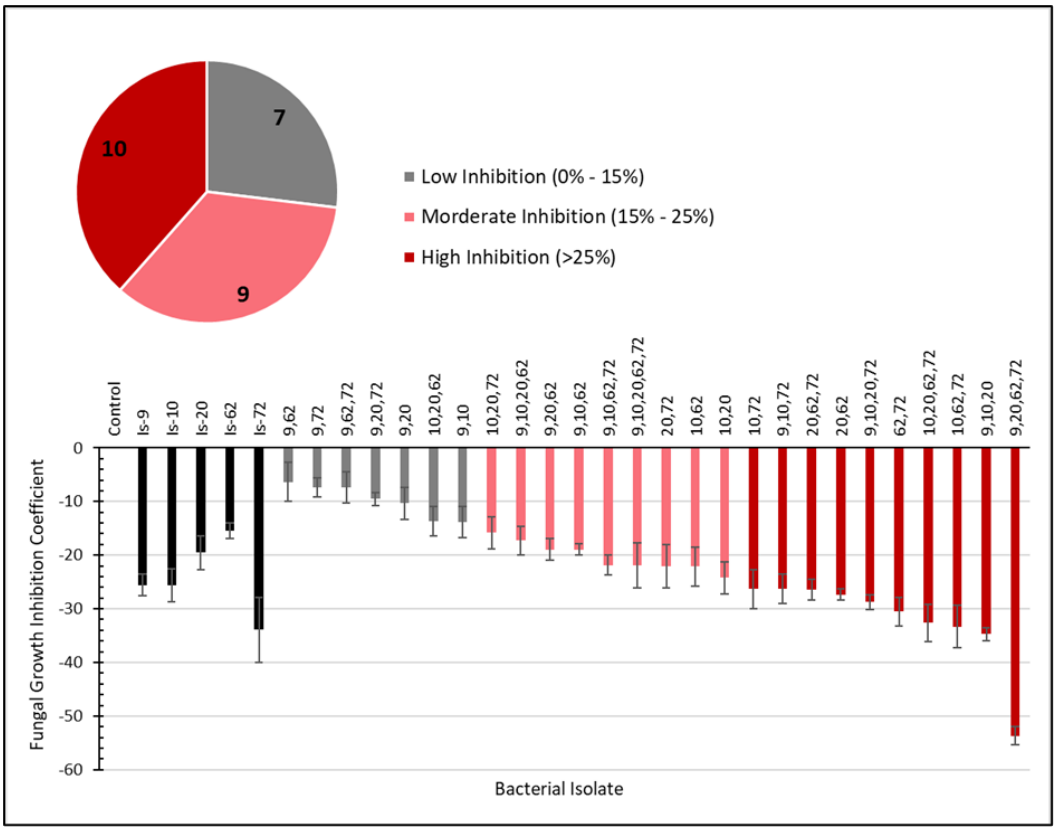


Figure 5: Correlation network between strains positively correlated with *Xylaria* (green edges) and negatively correlated (red edges). Node size indicates the average count in severe disease and edge size absolute correlation. Three communities of strains were detected using the GLay community detection algorithm.

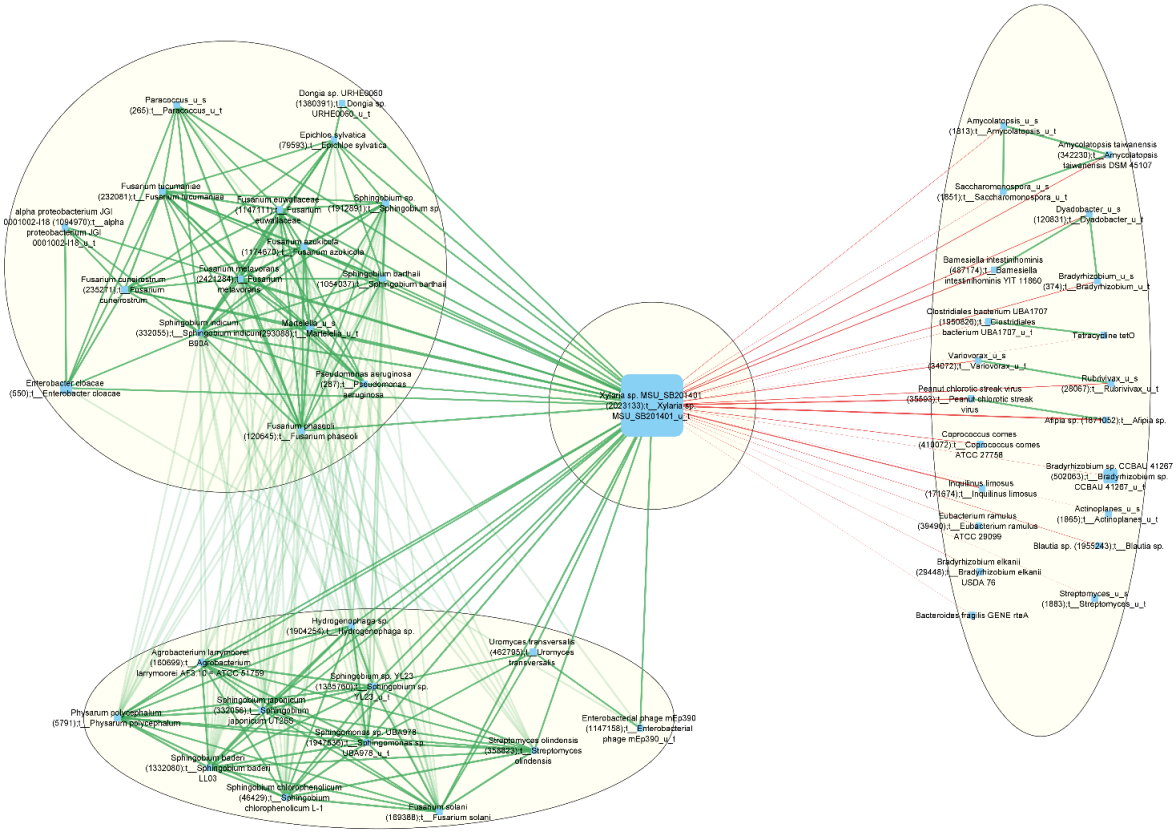


Figure 6: Principal component analysis of the metagenomics samples. Labels (as in Table 1) indicate what sample it corresponds to and the label size indicates the relative amount of Xylaria in the sample.

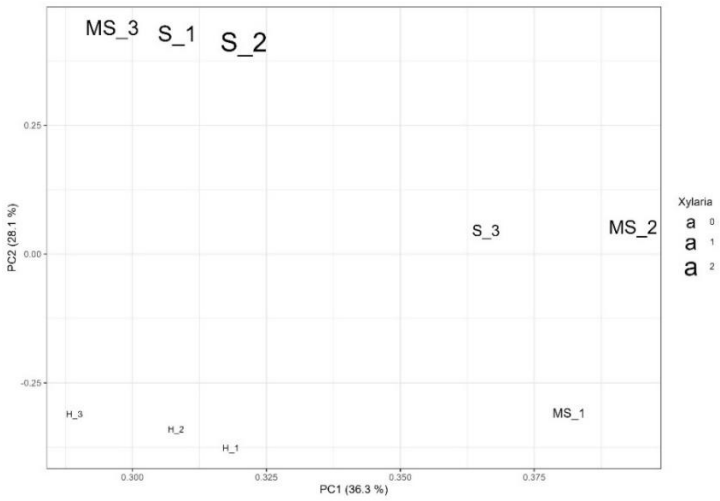
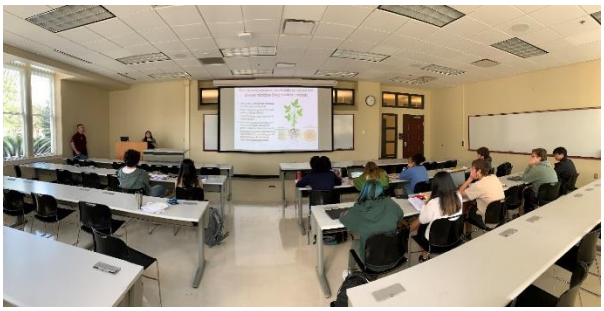


Figure 7. Quantification of images of plants from the field trial

A. Experimental design presentation



B. Field trial results presentation