CHARACTERIZE ROOT MICROBIAL COMMUNITIES WITH ANTI-FUNGAL ACTIVITIES IN SOYBEAN
PROJECT 53-2023
FINAL REPORT FOR 2020-2024 ACTIVITIES (MARCH 31ST, 2024)

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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 *Bacillus*. 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. **Assembly of a library of bacterial isolates -Soybean Root Endophyte Library (REL)-** from healthy soybean plants, collected from the MS Delta farms with farmers' agreement. The endophytic compartment of the soybean root microbiome was separated from the rhizosphere compartment; consequently, endophytic strains were isolated by cultivation on artificial media in the laboratory to assemble a Soybean Endophyte Library (REL). REL comprises approximately 230 isolates; 50% of REL strains have been tested for anti-Xylaria activity.
2. **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 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.
3. **Comparative metagenomic analyses of healthy and TRD symptomatic soybean.** We developed a statistical framework for analyzing the effect of *X. necrophora* and bacterial isolates on plant phenotypes in laboratory experiments. We developed models to discriminate antifungal effects from plant growth effects and to build predictive models of antifungal and growth effects from in vitro measurements. Cumulative results are shown in **Fig. 1** and **2**. Some of the results obtained from the comparative analyses have been published in the peer-reviewed journal *Microorganisms*.
4. ***In vitro* testing of beneficial isolates using co-cultivation assay:** The antagonistic activity of the soybean endophytic bacterial isolates from REL was tested against the fungal pathogen Xylaria sp. using an in vitro co-cultivation assay. We tested the anti-fungal activity of approximately 100 isolates. The cumulative results from these assays are shown in **Fig. 3** and **Table 1**.
5. **In planta testing of beneficial isolates using soil collected from agricultural fields and sterile Jiffy pellets.** We tested the anti-TRD effect of the endophytic strains identified as Xylaria sp. inhibitors in laboratory assays directly on soybean plants. For this, we developed and optimized a protocol for coating soybean seeds with beneficial isolates. Plants growth from endophyte-treated (alongside 'not treated' controls) were infected with Xylaria sp. The effect of the seed treatment on TRD was assessed by measuring the dry weight of the soybean canopy in 4-week-old plants. 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. 4A, B**). Soybeans treated with Is-62 (**Fig. 4C**) and Is-71 (**Fig. 4D**) displayed an improvement in overall development, demonstrating their capacity to promote plant growth.
6. ***In vitro* Bacterial-Fungal Cocultivation Assays with Synthetic Communities:** For a co-cultivation experiment using synthetic communities (syncomms), isolates with moderate to high

anti-*X. necrophora* and anti-TRD characteristics were selected. Isolates 9, 10, 20, 62, and 72 were used to construct 26 syncoms, including 1 of five isolates, 5 of four isolates, 10 of three isolates, and 10 combinations of two isolates. Seven syncomms inhibited X. necrophora growth by less than 15%, nine syncomms inhibited X. necrophora growth by 15% to 25%, and ten syncomms had the most substantial fungal inhibition effects of 25% and higher (**Fig. 5**).

7. ***In planta* testing of synthetic communities using soil collected from agricultural fields.** We tested the anti-TRD effect of eight syncomms selected from previous *in vitro* trials directly on soybean plants. The selected synthetic communities are as follows A[62, 72], B[9, 10, 20], C[10, 62, 72], D[20, 62, 72], E[9, 20, 62, 72], F[10, 20, 62, 72], G[9, 10, 20, 72], and H[9, 10, 20, 62, 72]. 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. 6A, B**).
8. **Modeling for 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 (**Fig. 7**). We generated a predictive understanding of how bacterial strains impact *Xylaria* accumulation over the disease progression (**Fig. 8**).
9. **A pilot field trial to test selected bacterial isolates for inhibitory activity against *Xylaria necrophora* was implemented in the third year.** The pilot field trial started on June 7, 2022. During the field trial, several plant measurements were collected to assess TRD disease phenotype (**Fig. 9**). 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 and harvest data were analyzed using statistical methodology to assess bacterial isolates effect.
10. **A comprehensive field trial was conducted in year four of the project**. Seed treatments were performed the week prior to the May 17, 2023 planting date. The soybeans used were Asgrow brand soybean 46XF3. Treatments were made by adding a concentrated bacterial solution to finely ground sterilized peat moss. This mixture was then coated to the seed using a high sucrose concentration solution. The seeds were dried, bagged, and transferred to the Delta Research Center in Stoneville, MS. The treatments consisted of eight bacterial isolates that showed antifungal properties to *Xylaria necrophora* as well as eight combinations of these isolates that showed promising protection *in vitro* and *in vivo*. The bacterial isolates used were 9, 10, 15, 20, 21, 62, 71, and 72. The synthetic communities (syncomms) are as follows A[62, 72], B[9, 10, 20], C[10, 62, 72], D[20, 62, 72], E[9, 20, 62, 72], F[10, 20, 62, 72], G[9, 10, 20, 72], and H[9, 10, 20, 62, 72]. The field trial also received a further spray treatment of the bacterial solutions closer to the reproductive stage of the soybean plants. The experiment was set up as a randomized complete block with 4 replications. Plots were established as 4 rows by 20 foot in length on 40 inch centers using an Almaco 4-row cone planter. Seeds were populated at 11 seeds per foot, which is slightly above the traditional planting rate to provide a more competitive/stressful environment that promotes disease incidence. The first 2 rows of the plot were inoculated with *Xylaria necrophora* infested millet seed sowed in-furrow at planting at 1 gram per row foot. The last two rows of the plot were left non-inoculated to compare treatments in both situations (**Fig. 10A B C D**).
11. **Metagenome analysis of treatment effects after the completion of the field trial**. We analyzed selected roots exhibiting TRD as well as roots of bacterially treated plants where some degree of protection was observed (**Fig. 10E F, Fig. 11**). We have selected samples from plants treated with Is-72 and syncomm-H for further analysis of endophytic bacteria and fungal communities; the

metagenome sequencing will be performed by CosmosID and bioinformatics analysis will be conducted in our lab.

12. **We analyzed the post-harvest results of the field trial** from 2023. Current results evaluate the total yield loss due to Xylaria infection in a soybean field and demonstrate the rescuing effect for two of the bacterial isolate treatments: Is-15, Is-71 (**Fig. 12**). It is remarkable that the rescuing effect almost entirely cancels the loss effect due to Xylaria infection. These results were obtained on single isolates cultivated in the same field from last year's trials. The syncomms were cultivated on a new field, and did not show major effects, potentially due to the lack of significant Xylaria infection. The disease incidence and severity were relatively low in both the isolate alone and the syncomms trials. With regards to disease severity in the syncomm inoculated plots, a 36% reduction was observed in plots treated with an additional soil spray of treatment D when compared to the control. We conclude that the results of the field trial demonstrate some rescuing effect of bacterial treatment and that additional work needs to be conducted to confirm the results.

Key research outcomes:

1. **Assembly of a library of bacterial isolates (REL).** Identification of bacterial isolates composition and characterization of X. necrophora inhibitory effect. Our collection of beneficial microbes constitutes a valuable resource for implementing sustainable strategies for Xylaria sp. control in the field in the coming years.
2. ***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.
3. **Metagenomics and microbial community dynamic analyses** to characterize the rhizosphere and endophytic bacterial and fungal communities in Mississippi soybean healthy plants and plants infected by *Xylaria necrophora*.
4. **Pilot and comprehensive field trials**: the pilot field trials were completed in 2022, with an Asgrow brand soybean, AG43XO. The soybeans field tails were completed in 2023 and used Asgrow brand soybean 46XF3. The experimental treatments included eight bacterial isolates and eight syncomm bacterial treatments.
5. **Models for predictive understanding of TRD development from metagenomics data**. We developed a predictive model for understanding how bacterial strains impact *Xylaria* accumulation over the disease progression.

Opportunities for training and professional development provided by the project:

1. Training of three postdocs, dr. Aline Bronzato-Badial (Biochemistry, MSU); dr. Xin Ye (Biochemistry, MSU); dr. Philip Berg (IGBB, MSU) and five graduate students: S. Nejat VanWarmerdan (graduated with PhD in 2021); Jasmine Uyen Wesser, (graduated with MS in 2022); Philip Berg (graduated with PhD in 2022); Joshua Mitchell (Biochemistry/Computational biology, MSU); Afra Bhuyian (Biochemistry, MSU).
2. Training of 13 undergraduate students in microbiology, molecular and plant biology techniques: Jasmine Wesser (Biochemistry, MSU); Griffin Emerson (U. of Wisconsin); Landon Hawk (Biochemistry, MSU), Samuel Buckley (Biomedical Engineering, MSU), Aja Black (Biochemistry, MSU), Josh Mitchell (Biomedical Engineering, MSU), Tyrikus Hayes (Biochemistry, MSU), Autumn Dodson (Medical Technology, MSU), Slade Smith (Biochemistry, MSU), Fenny Patel (Biochemistry, MSU), Pradeep B K (Biochemistry, MSU), Stephanie Dauber (Biochemistry, MSU) and Sean McGrath (Biochemistry, MSU).

Products and dissemination of results to communities of interest:

1. 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.
2. Filing an Invention Disclosure Form: IDF #:2020.1149 "*Identification of microbial strains for the biological control of the soybean fungal pathogen Xylaria sp.*" (12/2020).
3. Master's student thesis: 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. Video presentations at DREC meetings by the PI.
5. Poster presentations at the Americal Phytopathological Society (APS), Southern Section of the American Society of Plant Biologists (SS-ASPB), American Society of Plant Biologists (ASPB); oral presentations at the Southern Section of the American Society of Plant Biologists (SS-ASPB).
6. The project was entered into the National Soybean Research Database: https://www.soybeanresearchdata.com/Project.aspx?id=54983
7. An article featuring our project, "Soils May Hold Solutions for Taproot Decline," has been posted on the Soybean Research & Information Network (SRIN) website: https://soybeanresearchinfo.com/research-highlight/soils-may-hold-solutions-for-taproot-decline/

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 researched and deployed methods for biological control of *X. necrophora* to enhance soybean production by reducing TRD incidence. Our results provide evidence and proof-of-principle for the development of bacterial treatments and the design 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.*

FIGURES and TABLES:

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.

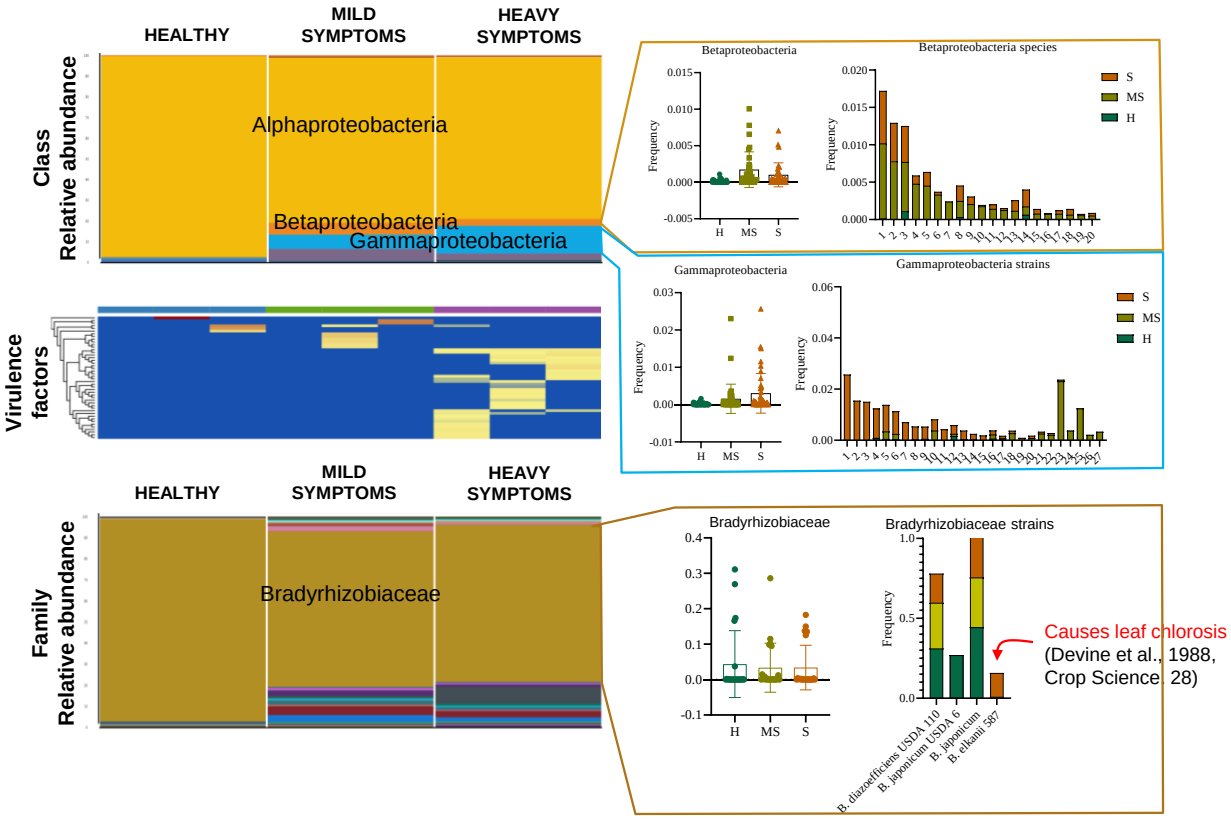


Figure 1: *Xylaria sp.* depletes N-fixation bacteria and increases virulent bacteria in soybean roots. Comparative analyses of endophytic bacterial communities of healthy soybean roots and plants with mild and heavy TRD symptoms. The charts show relative abundances of bacteria classes (top), bacteria containing virulence factors (middle), and bacterial families. The insets show representative bacteria classes and families with significant differences between healthy and diseased plants.

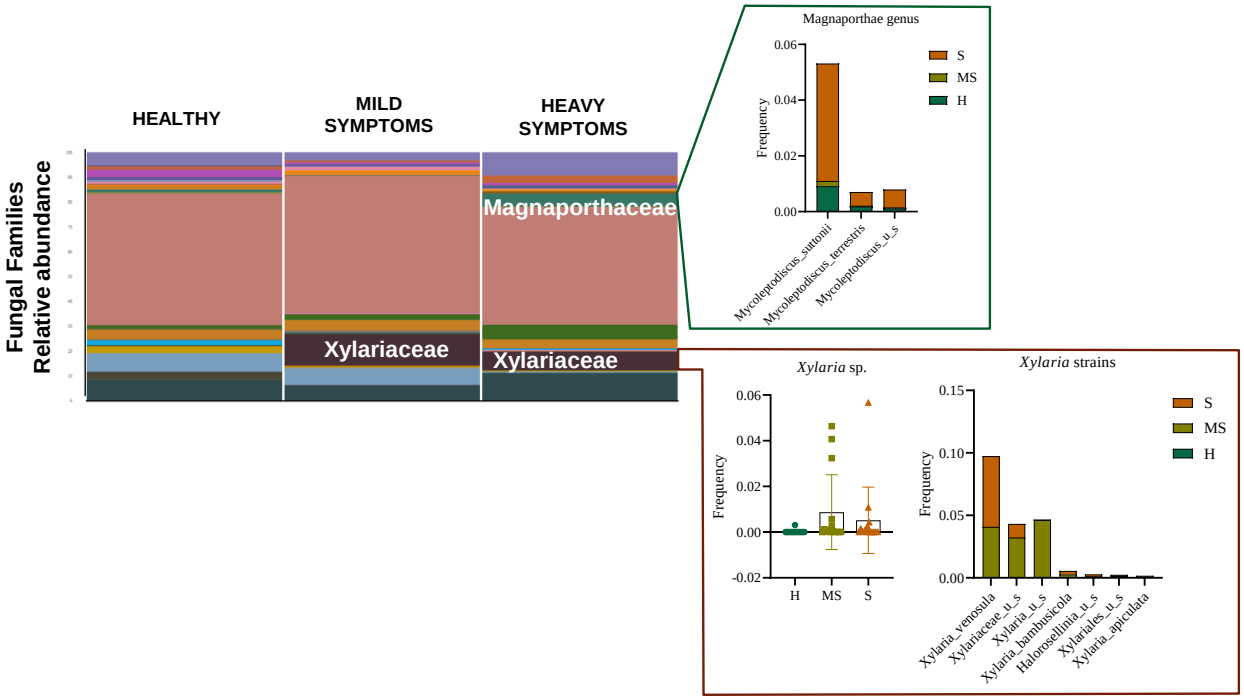


Figure 2: *Xylaria sp.* abundance increases in microbiomes of mild and heavily TRD symptomatic plants. Comparative analyses of endophytic fungal communities of heathy soybean roots and plants with moderate and heavy TRD symptoms. The charts show the relative abundance of fungal families. The insets show representative families with significant differences between healthy and diseased plants.

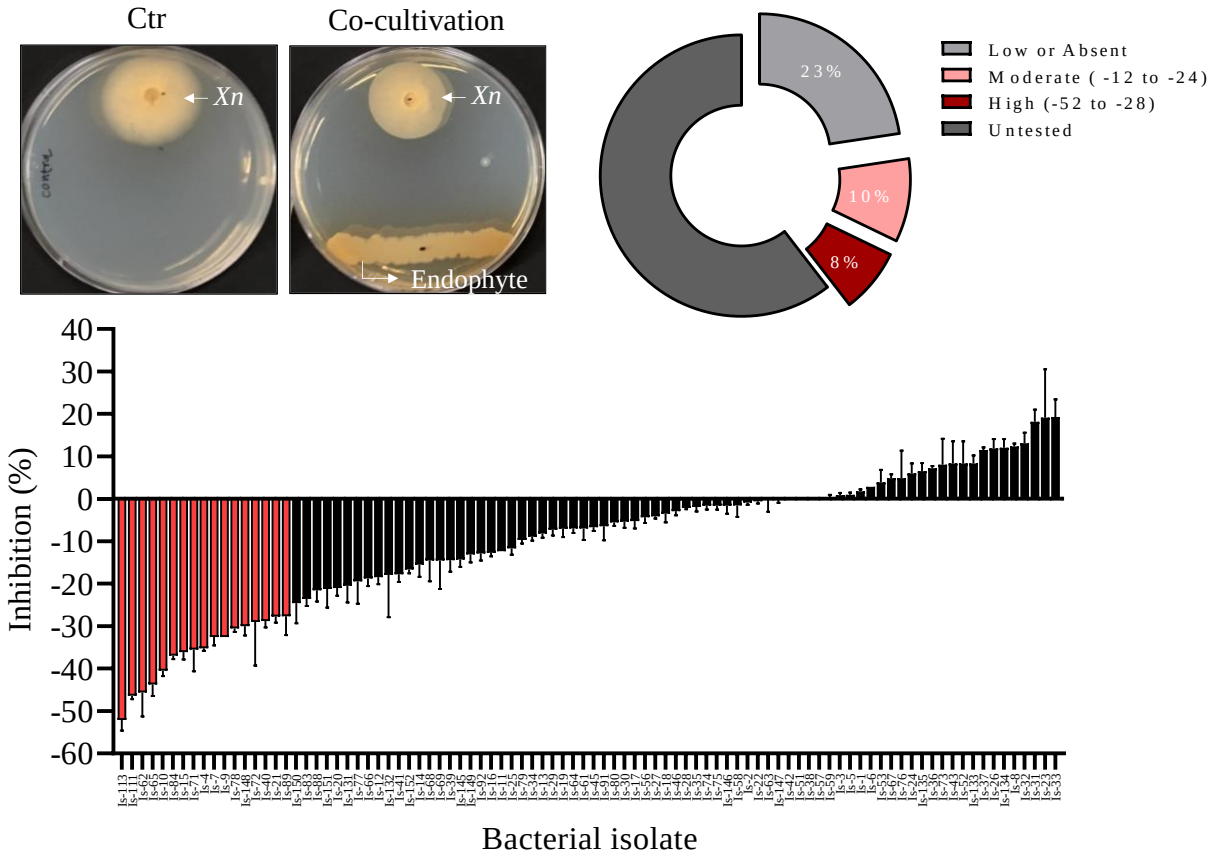


Figure 3: 92 bacterial isolates were tested in co-cultivation assays with Xylaria to identify isolates with anti-Xylaria properties.

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 4. Results of *in planta* screen of bacterial-treated soybean with and without Xylaria inoculation.

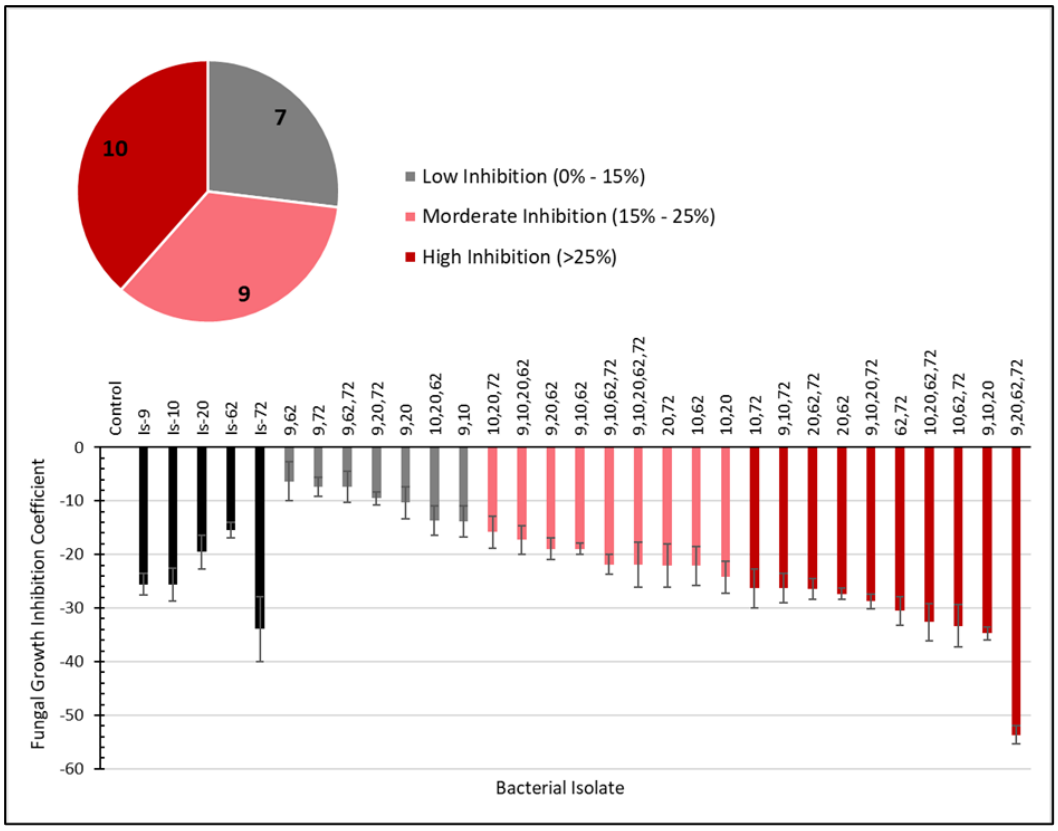


Figure 5. Bar graph displaying the percentage of inhibition by the 26 synthetic community (syncomm) combinations and 5 individual isolates against *X. necrophora*.

A)

B)

Figure 6. *In planta* trials of bacterial synthetic community-treated soybeans with (left row) and without (right row) Xylaria inoculation: A) in sterile soil; B) in MS Delta soil.

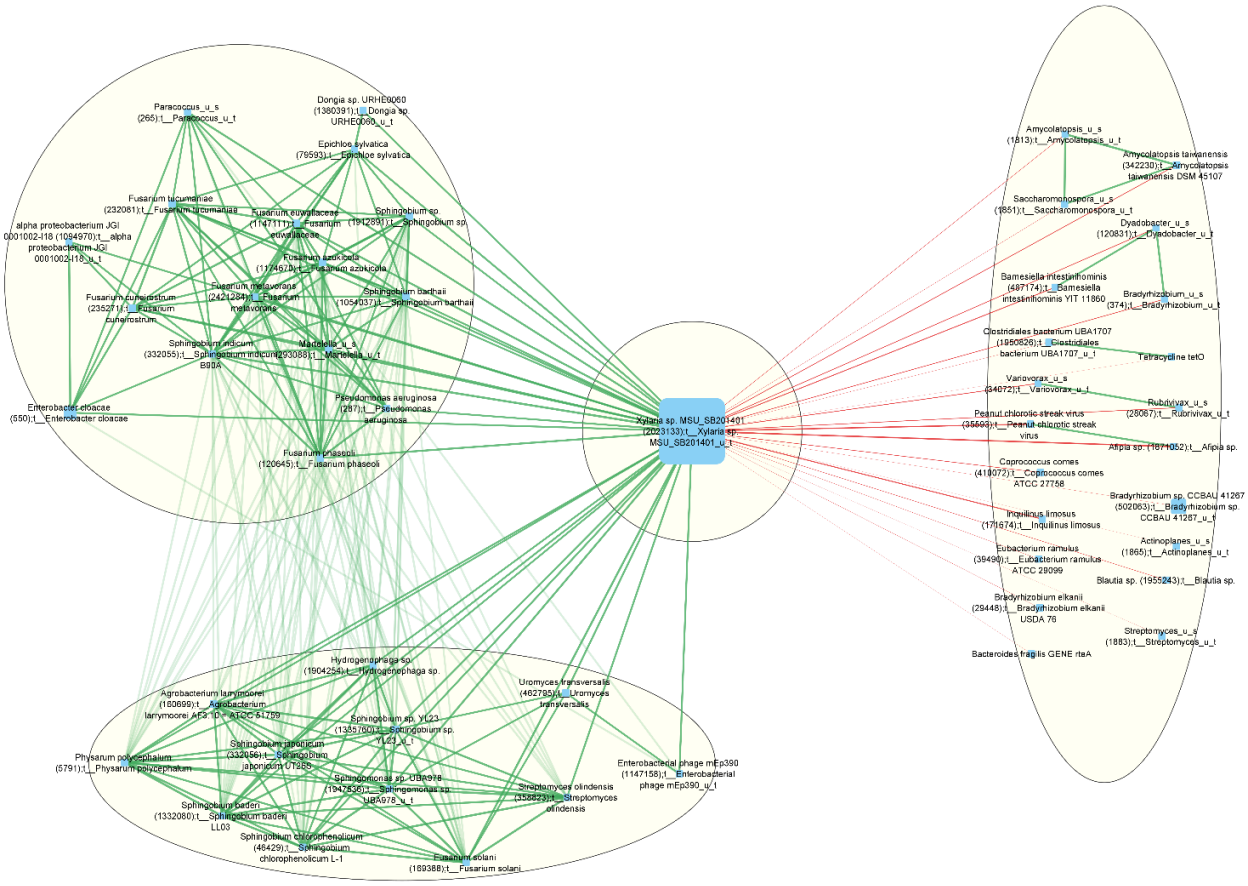


Figure 7. Correlation network between strains positively correlated with Xylaria (green edges) and negatively correlated (red edges). Node size indicates the average count in severe disease and edge size absolute correlation. Three communities of strains were detected using the GLay community detection algorithm.

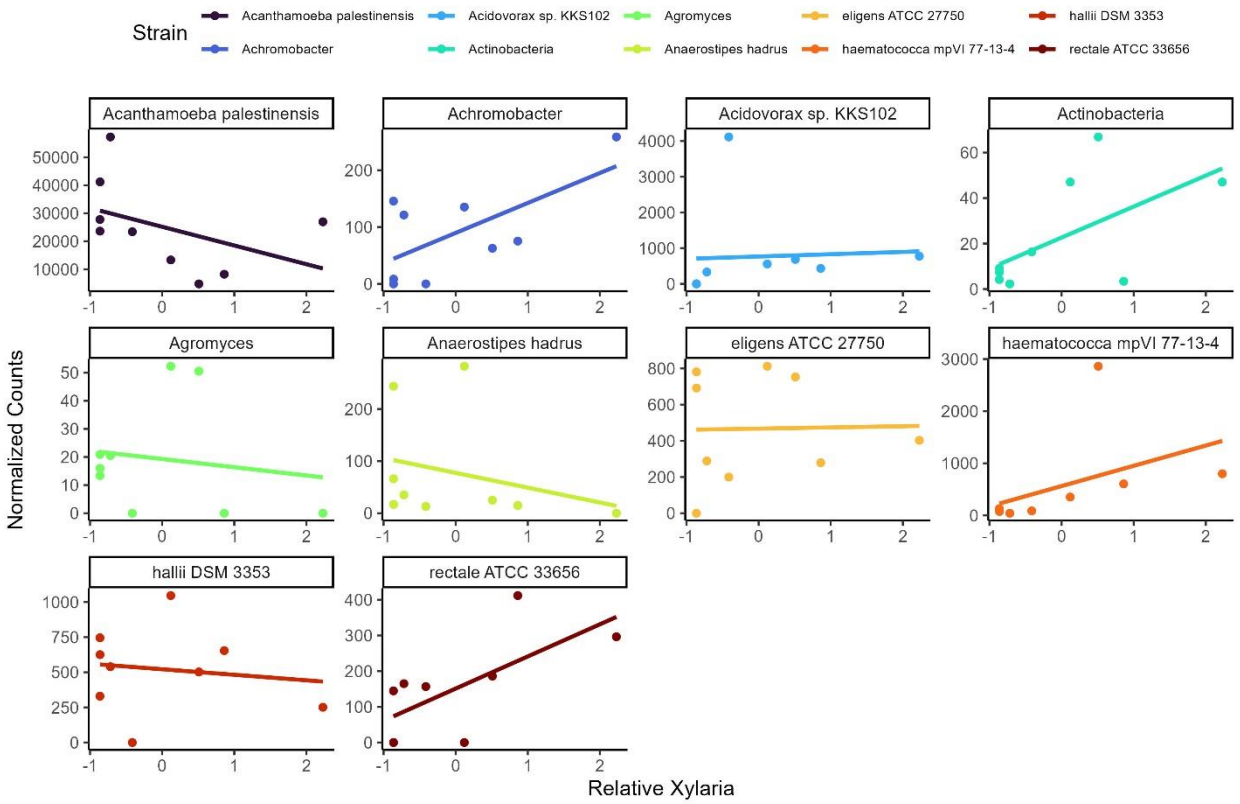


Figure 8. Relationship between the top predicted features and Xylaria. Colors and facets indicate different strains, and lines indicate a simple linear regression.

Figure 9. Images of plants from the pilot field trial. Images were taken on 07/12/2022, approximately one 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.

A

B

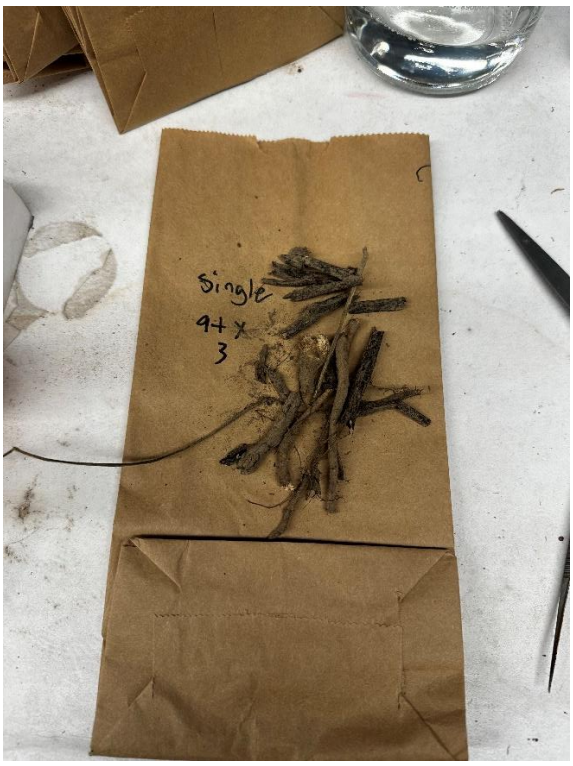
C

D

E

F

Figure 10. Images of plants from the 2023 field trial. Images were taken on September 2023, approximately 4 months after planting. **A, B** control, without and with Xylaria; **C, D** IS-9 treated, without and with Xylaria; **E, F**: IS-71 treated plants exhibiting signs of TRD.

B 

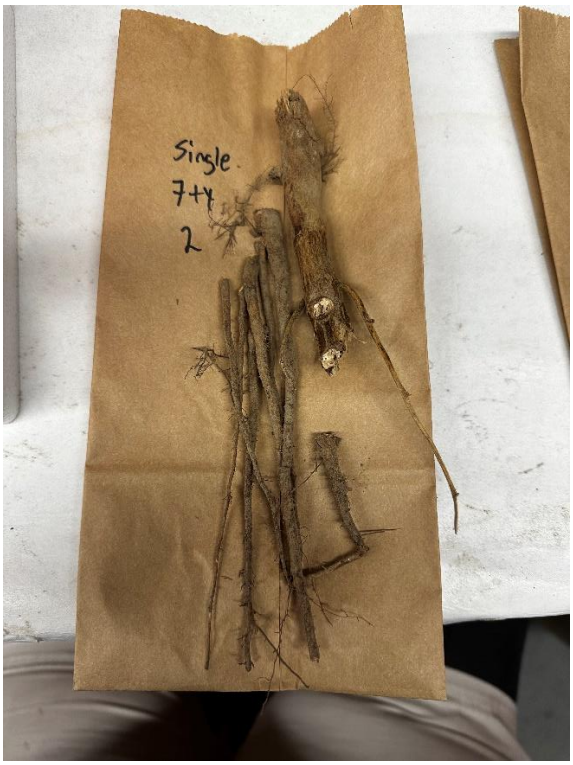
C 

Figure 11. Images of plants from the field trial. Images were taken on September 27 2023, approximately 4 and a half months after planting. **A:** control without Xylaria; **B:** IS-72 treated, with Xylaria inoculation; **C:** IS-62 treated, with Xylaria inoculation.

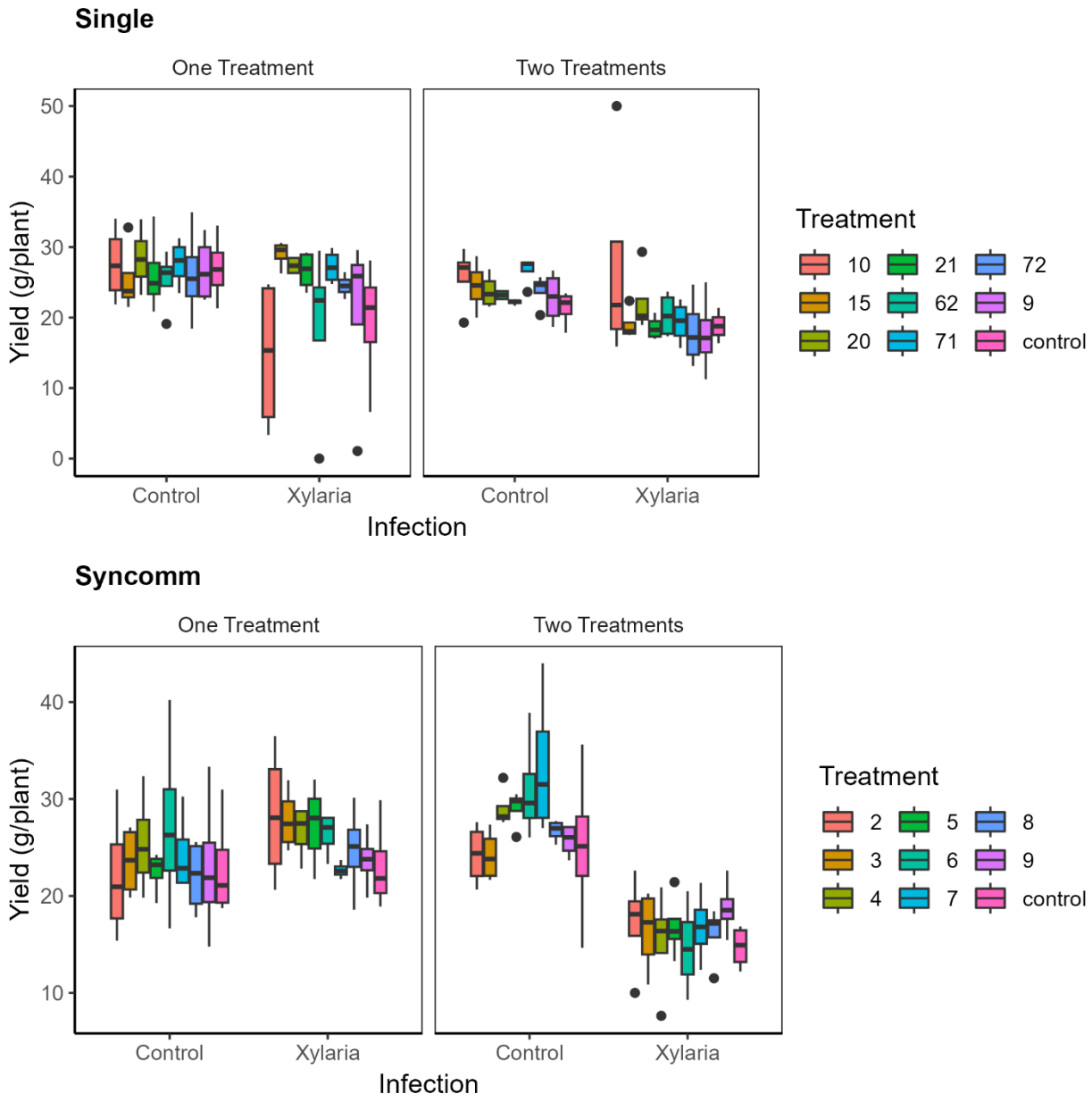


Figure 12. Field trial harvest results. Isolates and Syncomms effect in the presence or absence of Xylaria and with one or two treatments.