THESIS

A MICROBIOME APPROACH TO CULTIVATION AND MANAGEMENT OF SUGAR BEET

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ABSTRACT

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The world's population is projected to reach 9.8 billion by 2050, while the urgent threat of climate change is expected to impact crop physiology and pest dynamics. Understanding, preserving and leveraging the plant-associated microbiome can result in enhanced agroecosystem functioning and disease resistance for agricultural crops, thus improving food security. Sugar beet, an economically important sugar producer in the northern hemisphere, offers insights into plant-microbiome dynamics due to its susceptibility to pathogenic microbes and its association with disease suppressive soils. Cultural and chemical management practices of sugar beet are a persistent debate due to the potential negative effects on the essential microbiome and the emergence of resistant populations. To investigate the impact of weed control strategies on the soil microbiome, we conducted a long-term field study at two locations. Using next-generation sequencing and in vitro assays, we assessed the effects of glyphosate, a mix of selective herbicides and tillage treatments on the structure and function of the soil microbiome. Furthermore, we isolated 136 bacteria from the sugar beet agroecosystem and evaluated their antagonistic abilities against key diseases of sugar beet. Through in vitro and greenhouse assays, we identified effective microbial consortia for disease reduction. Additionally, we investigated the interactions between a single antagonistic isolate and an important fungal disease of sugar beet using transcriptomic analysis to reveal underlying mechanisms for biological control and

pathogen response. This comprehensive understanding of the impact of various management strategies on the microbiome provides crucial insights for future crop management and highlights the potential for exploiting beneficial microbes to enhance disease control.

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THESIS INTRODUCTION

The world population is expected to increase to 9.8 billion people by 2050 (Food and Agriculture Organization of the United Nations, 2018) and agricultural crop production will be required to sustain the population while simultaneously reducing its environmental footprint for future generations (Foley et al., 2011). The imminent threat of climate change will lead to shifts in crop physiology, pest lifecycles, and microbial communities (Delgado-Baquerizo et al., 2020; Malhi et al., 2021). Understanding and harnessing the soil microbiome can lead to enhanced agroecosystem functioning, consequently promoting soil health and crop resistance to new biotic and abiotic stresses (Dubey et al., 2019). Worldwide food security depends on agricultural research and innovation to advance effective, sustainable farming practices.

Crop-associated soil microbiota play a crucial role in promoting plant growth and improving crop yield. Microbes can enhance nutrient acquisition from the soil (Abdiev et al., 2019; Bakhshandeh et al., 2020; Richardson & Simpson, 2011; Xu et al., 2022), increase tolerance to abiotic stresses (Abdiev et al., 2019; Ghosh & Dutta, 2016; Moradtalab et al., 2020; Shahid et al., 2021; Wang et al., 2021; Yang et al., 2021) and improve resistance to disease (Berg et al., 2005; Howell et al., 2000; Khastini et al., 2012; Shoresh et al., 2010). Plants and their microbiota are closely linked and form a holobiont (Vandenkoornhuyse et al., 2015), which can be conserved across members of the species (Hamonts et al., 2018). Roots secrete primary and secondary metabolites which help to recruit the specific species present in the crop rhizosphere (Badri et al., 2013; Cardoso et al., 2014; Jacoby et al., 2020). Recruitment can also come from signaling by the plant (Santoyo, 2022), as well as microbe-microbe signaling such as

quorum sensing (Chamkhi et al., 2020; Cortez et al., 2022; Venturi & Keel, 2016). Plants have developed a complex immune response to decipher beneficial microbes from pathogens (Hacquard et al., 2017; Zipfel, 2014), enabling colonization by endophytic bacteria and mycorrhizal fungi (Trivedi et al., 2020). Under stress conditions such as drought or pathogen attack, plants have been observed to associate with specific microbial communities (Liu et al., 2021; Trivedi et al., 2022). A deeper understanding of the complex interactions taking place within the crop-associated microbiome could lead to the discovery of novel agricultural management strategies.

Sugar beet accounts for about 30% of the worldwide sugar production and the crop is being studied as a major producer of ethanol (Dohm et al., 2014; Maung & Gustafson, 2011). Sugar beet, a C3 plant, grows in temperate regions of Europe, North Africa, Asia, and North America, compared to the C4 alternative, sugar cane, which is mainly grown in tropical regions (Cooke & Scott, 2012).The two types of commercial sugar are very similar, however growing and processing procedures vary based on crop. Sugar beet is a biennial root crop and grows for two seasons before flowering, however it is harvested for sugar after the first year (Kuroda et al., 2023). Crop yield is based on the sucrose content in the roots and is therefore essential for growers to cultivate healthy plants. Genetic variation can impact sucrose quantity and quality (Biancardi et al., 2010), however environment and management has been observed to have a greater influence (Hoffmann, 2010; Hoffmann et al., 2021).

Sugar beet cultivation involves a variety of management strategies, both cultural and chemical, that have potential to alter the agroecosystem. Weed and disease management are two important aspects of sugar beet cultivation that could directly

impact the plant associated microbiome. Frequent herbicide use is common due to the crop's slow emergence and delayed canopy development (Gerhards et al., 2017; Hoffmann et al., 2021). The impact of chemical herbicides in agricultural fields on non-target microorganisms and soil ecosystems has been an important debate in recent years (Puigbò et al., 2022; Ruuskanen et al., 2023), however variability in experimental design has led to inconsistent results. Managing for diseases is also important for a healthy crop and has traditionally used pesticides for control. The sugar beet associated soil microbiota can be associated with disease suppression in certain soils and promotion of plant health through nutrient uptake and stress tolerance (Carrión et al., 2018, 2019; Mendes et al., 2011). Therefore, the sugar beet serves as a model crop for understanding microbiome-based disease management (Wolfgang et al., 2020). Due to the importance of the soil microbiome in promoting crop health and suppressing disease, the influence of management strategies on sugar beet could have significant consequences for crop yield and quality.

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CHAPTER 1: HERBICIDES HAVE MINIMAL AND VARIABLE EFFECTS ON THE STRUCTURE AND FUNCTION OF BACTERIAL COMMUNITIES IN AGRICULTURAL SOILS

INTRODUCTION

Over the last five decades, weed management in the United States (U.S.) has relied mainly on synthetic herbicides. It is estimated that 87 million hectares of agricultural land are treated with herbicides, and these applications are necessary to avoid revenue losses of $15.5 billion annually (Gianessi & Reigner, 2007). Traditional mechanical weed control measures can be inefficient and expensive, potentially leading to crop damage or losses (Machleb et al., 2020; Monteiro & Santos, 2022). Soil disruption caused by tillage equipment can bring dormant seeds to the surface and possibly spread perennial weeds through the distribution of propagules (Cioni & Maines, 2010). Herbicides are classified based on their selectivity, mode of action, and translocation in the plant (Duke & Dayan, 2022; Vats, 2015). Selective herbicides only target particular weed species through specific biochemical pathways, which can require multiple products to be applied to account for the diversity of weeds (Beffa et al., 2019; Dayan, 2019; Morishita, 2018). Careful timing and selection of selective herbicides are necessary to gain proper control throughout the agricultural season (Bhadra et al., 2020;Jursík et al., 2008; Carey & Kells, 1995). Due to their popularity and increased use in agricultural systems, the impacts of continued use of these chemicals on human and animal health, non-target plants, microorganisms and the environment has been under investigation (Puigbò et al., 2022; Ruuskanen et al., 2023).

The soil microbiome plays a critical role in soil functioning and is essential for plant growth and health (Singh et al., 2020; Trivedi et al., 2021). Herbicides are postulated to profoundly impact ecosystem functions via altering soil microbiome either directly by impacting survival or function (Darine et al., 2015; Chávez-Ortiz et al., 2022; Valle et al., 2006) or indirectly by altering host function (Mohamed et al., 2021;Fuchs et al., 2021; Lu et al., 2019). For example, herbicides are reported to have an impact on microbial community structure which has the potential to change microbial functioning (Tanney & Hutchison, 2010; Zobiole et al., 2011; Cycoń et al., 2012; Cycoń et al., 2013; Newman et al., 2016). However, the effects are rate dependent, and most studies indicate that when applied at or below the recommended field-rate herbicides exerts negligible (Ratcliff et al., 2006;Barriuso et al., 2011; Lane et al., 2012; Rosenbaum et al., 2014)) or minor (Weaver et al., 2007;Barriuso et al., 2010; Barriuso & Mellado, 2012) effects on microbial community structure, and negligible effects on functionality (Ratcliff et al., 2006). Inconsistent results are not surprising given the strong influence of location and soil characteristics on microbial communities and the generally small effect of herbicide treatments at a field scale. Moreover, the limited resolution of the techniques used to determine the impacts of herbicides on microbial community composition or diversity may have contributed to the variability (Tanney & Hutchison, 2010; Newman et al., 2016; Arango et al., 2014).

Weed control is an essential cultural management strategy for sugar beets. Without it, yield losses could reach up to 83%, leading to a monetary loss of approximately $1.25 billion for U.S. growers (Soltani et al., 2018). As sugar beets are slow to emerge and have delayed canopy development, chemical application programs

are necessary from crop emergence until the 8-leaf stage (Gerhards et al., 2017;Hoffmann et al., 2021). Non-selective herbicides, specifically glyphosate, have been increasingly applied since the appearance and dominance of resistant genetically modified sugar beets (Kniss, 2010; Stevanato et al., 2019). Several studies have reported the importance of soil microbiota in promoting disease resistance against a range of sugar beet pathogens (including *Rhizoctonia solani, Fusarium* sp., and *Pythium* sp.) and sustaining sugar beet yields via improved nutrient uptake and stress tolerance (Mendes et al., 2011;Carrión et al., 2019;Carrión et al., 2018;Chapelle et al., 2016; Postma & Schilder, 2015; Watanabe et al., 2011; Du et al., 2022). Due to the importance of the soil microbiome, the consequences of frequent herbicide applications on the microbial structure and function for sugar beets could significantly impact cultural management strategies. However, to the best of our knowledge, there is no report on the effects of herbicides on soil microbial community and functioning using well-replicated, properly controlled field studies at different locations.

This research aims to elucidate the impact of the repeated application of glyphosate, a mixture of selective herbicides, and tillage on soil bacterial communities under field conditions over two locations. Using amplicon sequencing, we first explored the impact of herbicide application on the structure and composition of soil bacterial communities. We then measured soil enzymatic activities, microbial respiration, and abundances of several genes involved in nutrient cycling to evaluate the impact of herbicide on microbial-mediated functions. We further performed in-vitro assays to assess the effect of glyphosate application on the growth of soil bacterial isolates. Overall, the relationship between microbes and herbicides is complex, and our findings

related to the effect of herbicides on soil microbial community are inconclusive. To better understand herbicides' ecological and evolutionary consequences in agroecosystems, we need more well-replicated, field-realistic, and long-term experiments using active ingredients and various commercial formulants.

MATERIALS AND METHODS

Experimental design

We established two field experiments in sugar beet plots at Lingle, Wyoming and North Platte, Nebraska. Both sites employ tillage management. In each site, twelve experimental plots were established in four replicate blocks with three treatments, glyphosate, mixed selective herbicide, and control (tillage), randomly allocated to plots in each block (Table 1.1). The glyphosate treatment consisted of three applications of the nonselective herbicide at a rate of 32 fl oz/acre at the 2 tiller, 6-8 tiller, and canopy growth stages. The mixed selective herbicide combined a pre-emergence herbicide, Nortron SC (ethofumesate), followed by three applications of Betamix (phenmedipham, desmedipham), Upbeet (triflusulfuron methyl), and Stinger (clopyralid) at the cotyledon, 2 tiller and 4-6 tiller stages. The 4-6 tiller application also included Warrant (acetochlor). Rates of the specific herbicides are listed in Table 1.1. The cultivation treatment plots were tilled and hand weeded twice, at the 4-6 tiller growth and 6-8 tiller stage.

Table 1.1 Herbicides, active ingredients, rates, formulations, and timings for applications of herbicides for treatments 1 and 2, and mechanical weeding schedule for treatment 3.

Treatment	Type	Treatment Name	Active Ingredient	Concentration	Formulation	Rate	Timing
1	HERB	Roundup PowerMax	Glyphosate	4.5 LBAE/GAL	SL	32 fl oz/a	2 TL
	HERB	Roundup PowerMax	Glyphosate	4.5 LBAE/GAL	SL	32 fl oz/a	6-8 TL
	HERB	Roundup PowerMax	Glyphosate	4.5 LBAE/GAL	SL	32 fl oz/a	Canopy
2	HERB	Nortron SC	Ethofumesate	4 LBA/GAL	SC	32 fl oz/a	PRE
	HERB	Betamix	Phenmedipham (0.65), Desmedipham (0.65)	1.3 LBA/GAL	EC	16 fl oz/a	Cot
	HERB	Upbeet	Triflusulfuron methyl	50%	DF	0.25 oz/a	Cot
	HERB	Stinger	Clopyralid	3 LBA/GAL	SL	2.4 fl oz/a	Cot
	ADJ	MSO	NA	100%	SL	1.5% v/v	Cot
	HERB	Betamix	Phenmedipham (0.65), Desmedipham (0.65)	1.3 LBA/GAL	EC	16 fl oz/a	2 TL
	HERB	Upbeet	Triflusulfuron methyl	50%	DF	0.25 oz/a	2 TL
	HERB	Stinger	Clopyralid	3 LBA/GAL	SL	2.4 fl oz/a	2 TL
	ADJ	MSO	NA	100%	SL	1.5% v/v	2 TL
	HERB	Betamix	Phenmedipham (0.65), Desmedipham (0.65)	1.3 LBA/GAL	EC	16 fl oz/a	4-6 TL
	HERB	Upbeet	Triflusulfuron methyl	50%	DF	0.25 oz/a	4-6 TL
	HERB	Stinger	Clopyralid	3 LBA/GAL	SL	2.4 fl oz/a	4-6 TL
	ADJ	MSO	NA	100%	SL	1.5% v/v	4-6 TL
	HERB	Warrant	Acetochlor	3 LBA/GAL	CS	1.25 qt/a	4-6 TL
3	CULT	Tillage					4-6 TL
	CULT	Handweeding					4-6 TL
	CULT	Tillage					6-8 TL
	CULT	Handweeding					6-8 TL

Sample collection and DNA extraction

Samples were collected at the same six timepoints for all treatments, across all plots. If an herbicide was applied, sampling was completed 3-4 days after an application. Bulk soil samples were collected at pre-emergence, cotyledon emergence, 2-4 leaf stage, 6-8 leaf stage and canopy cover. At each sampling timepoint, we combined three soil cores per plot taken at a depth of 0 to 7.5 cm. Samples from each plot were mixed thoroughly and shipped on ice to Colorado State University. Field moist soil was immediately passed through a 5.6 mm sieve for biological analysis and air-dried and sieved (<2 mm) for chemical analysis. Aliquots of soil samples were stored at -80 °C for subsequent DNA extraction. DNA was extracted from soils using the DNeasy Powersoil Kit (MO BIO Laboratories, Carlsbad, CA, USA) as per manufacturer's instruction. Extracted DNA was quality checked by NanoDrop 2000 (Thermo Fisher

Scientific, Waltham, Massachusetts, USA) and quantity checked by Qubit Fluorometer (Thermo Fisher Scientific) using Quant-iT dsDNA BR Assay Kit (Invitrogen, Carlsbad, CA, USA), and stored at −80 °C.

Amplicon sequencing and bioinformatic analyses

Bacterial 16S rRNA gene V4-V5 hypervariable regions were PCR amplified using primers 515F/806R (Caporaso et al., 2012) combined with adapter sequences and barcode sequences. Purified amplicons were sequenced at the Next Generation Sequencing Facility, Colorado State University on an Illumina MiSeq platform (Illumina Inc., San Diego, CA). Bioinformatics processing was performed using a combination of USEARCH (Edgar, 2010) and UNOISE3 (Edgar, 2016). Operating taxonomic unit (OTU) tables based on 97% sequence similarity were generated using the USEARCH pipeline. Sequencing run quality was assessed using fastQC (Andrews, 2010). The raw sequences were discarded if they contained ambiguous nucleotides, had a low ($Q < 20$) quality score, or were short in length (<100 bp). Adapters and primers were removed using cutadapt (Martin, 2011). Then samples were demultiplexed. Paired-end reads were merged, and quality was assessed with an initial quality check test. The representative set database was created using the UPARSE algorithm (Edgar, 2013). Unique sequences were located and sorted into unique OTUs. OTUs were clustered using DADA2 and DeNoised using uNoise3 (Xiong, He, et al., 2021) as described in Xiong et al., (2021b). OTU tables were generated by mapping reads to the representative set database. OTUs were counted at the sample level. Taxonomic identification of bacteria was obtained against the Silva database (Pruesse et al., 2007). Bacterial sequences that match host mitochondria and chloroplast were removed.

Soil enzymatic activities

β-D-cellubiosidase (CB), α-Glucosidase (AG), β-glucosidase (BG), N-acetyl-β-Glucosaminidase (NAG), Phosphatase (PHOS) and β-Xylosidase (XYL) activities were measured using 4-methylumbelliferyl (MUB) substrate yielding the highly fluorescent cleavage products MUB upon hydrolysis. All the enzyme assays were set up in 96-well microplates as described by Bell et al. (2013). Twelve replicate wells were set up for each sample and each standard concentration. The assay plate was incubated in the dark at 25 °C for 3 h to mimic the average soil temperature. Enzyme activities were corrected using a quench control. Fluorescence was measured using a microplate fluorometer (EnSpire 2300 Multilabel Reader, Perkin Elmer, Waltham, MA, USA) with 365-nm excitation and 460-nm emission filters. The activities were expressed as nmol h−1 g−1dry soil. Enzyme activities were calculated for soil samples from two sites collected at all the time points.

Soil respiration analysis

Soil respiration was measured using MicroResp^{TM} (Macaulay Scientific Consulting, UK). Approximately 350 mg of soil was added to deep well microtiter plates to which 30 μl of water was added in each well. A rubber sealing mat was used to seal the deep well plate to an indicator plate, and plates were incubated in the dark over 6 h at 25°C as previously. After incubation, the CO_2 production rate was calculated based on the change in absorbance (A_{570}) of the indicator plate (Campbell et al., 2003).

Quantitative real-time PCR (qPCR) analysis

qPCR was performed to determine gene copy numbers for total bacteria and functional genes involved in nitrogen fixation (*nifH* encoding dinitrogenase reductase); denitrification (nirK and nirS encoding the cd1 and copper nitrite reductase; nosZ encoding the nitrous oxide reductase); phosphorous mineralization (phoC encoding for acid phosphatase); cellulose degradation (GH11 encoding for xylanase); and chitin degradation (GH18 encoding for chitinase). qPCR was conducted using primers and cycling conditions described previously (Trivedi et al., 2012; Fraser et al., 2017; Wang et al., 2022). qPCR reactions were carried out on extracted soil DNA from timepoints 1, 3 and 6 using Absolute qPCR SYBR green mixes (Qiagen Inc., Valencia, CA, USA) on an ABI Prism 7500 Sequence detection system. Standard curves for real-time PCR assays were developed by PCR amplifying the respective genes by their specific primers, PCR products purification using a PCR cleanup kit (Axygen Bioscience, Union City, CA, USA), cloning into the pGEM-T Easy Vector (Promega Corp.), and transformation into *Escherichia coli* JM109 competent cells (Promega Corp.) (details in(Trivedi et al., 2012)). Tenfold serial dilutions (10^8–10^1 copies per µl) of the plasmid DNA were subjected to a qPCR assay in triplicate to generate an external standard curve and to check the amplification efficiency. Standard curve regression coefficients were consistently above 0.99 and melt curve analysis verified a single amplicon per reaction in all the cases. Samples and standards were assessed in at least two different runs to confirm reproducibility of the quantification. Target copy numbers for each reaction were calculated from the standard curve and were used to ascertain the number of copies per µg of DNA.

In Vitro Glyphosate Assay

We used 86 bacteria previously isolated from sugar beet agroecosystems to evaluate the direct effect of the active ingredient glyphosate *in vitro*. Bacterial strains were characterized through Sanger sequencing (Eurofins Genomics, Louisville, Kentucky, USA) on the amplified 16S rRNA using primers 27f-1492r (Kanagawa, 2003). The bacteria were initially isolated on nutrient agar medium and stored as glycerol stock in the -80C freezer. Bacterial strains were revived twice on nutrient agar from glycerol. Isolated bacterial colonies were then inoculated into fresh nutrient broth, the stock was homogenized by vortexing and adjusted to 1 x 10^8 cells. Bacterial nutrient broth stock was added to a 1.1 mL 96-well plate with 10 mMol glyphosate dissolved in nutrient broth. Positive control wells containing bacteria without glyphosate and negative controls without glyphosate or bacteria were included on each plate. Three replications were performed for each bacterial strain. The plates were incubated at 30C for 48 hours and absorbance of the plates were read at 600 nm using a microplate reader (Tecan Infinite Pro 200, Mannedorf, Switzerland) and analyzed in the Tecan iControl software (Tecan, Crailsheim, Germany). Percent growth inhibition was calculated by comparing the absorbance of bacteria exposed to glyphosate and the positive controls:

$$\% \text{ Inhibition} = \frac{Abs_{control} - Abs_{treatment}}{Abs_{control}} \times 100$$

Statistical analysis

For analyzing amplicon sequence data, samples were rarified to the lowest occupancy of 10,000 reads. We used the R package ‘mctools’ to analyze microbial

community structure (Leff, 2017). To examine alpha diversity, Shannon diversity indexes were calculated and evaluated through general linear models (GLMs). Tukey HSD tests were used to determine influence of the above variables on alpha-diversity. Scatter plots were constructed in ‘ggplot2’ using Shannon’s diversity metric and correlated to actual sampling date (Wickham, 2016). Permutational multivariate analysis of variance (PERMANOVA) models were generated to determine significant beta-diversity differences correlating with timepoint and treatment. Bray–Curtis dissimilarity distances were calculated then ordinated in non-metric multidimensional scaling plot (NMDS) analysis by treatment for each timepoint. Scatter plots were further constructed in ‘ggplot2’ to visualize temporal changes using Axis 1 from the NMDS ordination plots for each timepoint. Statistical analysis of soil enzyme activity in different treatments were analyzed using a one-way ANOVA. Scatter plots were constructed in relation to sampling date. Ct values from qPCR data were normalized to a standard curve and calculated to gene copies per ng of DNA based on DNA concentration. Boxplots were constructed and significance was evaluated based on Tukey’s HSD. Volcano plots used to visualize significantly enriched or depleted taxa for treatments were constructed using ‘DESeq2’ (Love et al., 2014). A significance level of $\alpha = 0.05$ was used for all biomarkers evaluated in this study. All statistical analyses were completed using R v 4.0.5 (R Core Team & Team, 2022).

The visualization of the in vitro effects of glyphosate on the bacteria isolates was performed using the interactive tree of life (iTOL) interface (Letunic & Bork, 2021). First the bacterial phylogenetic tree was constructed using Clustal Omega by EMBL-EBI (Madeira et al., 2022). The tree and the metadata files derived from the in vitro assays

were imported into iTOL interface. In the iTOL output the growth for individual strains is displayed as percent growth inhibition. Significance was determined through a two-tailed student's t-test with a significance level of $\alpha = 0.05$.

RESULTS AND DISCUSSION

Herbicide applications have minimal impact on bacterial community structure

Our study provides evidence that differences in microbial structure and function are temporal, and variable based on environment, with minor differences based on herbicide treatments. In the experimental site in Lingle, WY, the impact of timepoint on alpha diversity was significant ($p < 0.001$), however no significant difference was found for either treatment compared to control (Supplementary Figure 1.1). In the North Platte, NE site, both timepoint ($p < 0.001$) and treatment ($p < 0.001$), as well as the interaction between timepoint and treatment, ($p < 0.001$), significantly impacted the alpha diversity. However, the treatment mediated impacts were more apparent in the later timepoints (Figure 1.1). Specifically, we observed a decrease in Shannon's diversity for the control plot, 6.58, compared to the glyphosate, 7.11, and mixed selective herbicide, 7.05, sites. Potential weed seed presence of species like kochia in the non-treated plots could increase antimicrobial allelopathic chemicals in the soil, leading to a decrease in microbial abundance and diversity (Geddes & Sharpe, 2022; Houlihan et al., 2019). Another possible reason for the decrease could be attributed to dead plant debris remaining on the soil after applications in both treatment plots, rather than being removed through mechanical weeding, and acting as a nutrient source that could support the microbial community (Schlatter Daniel et al., 2017; Rose et al., 2016).

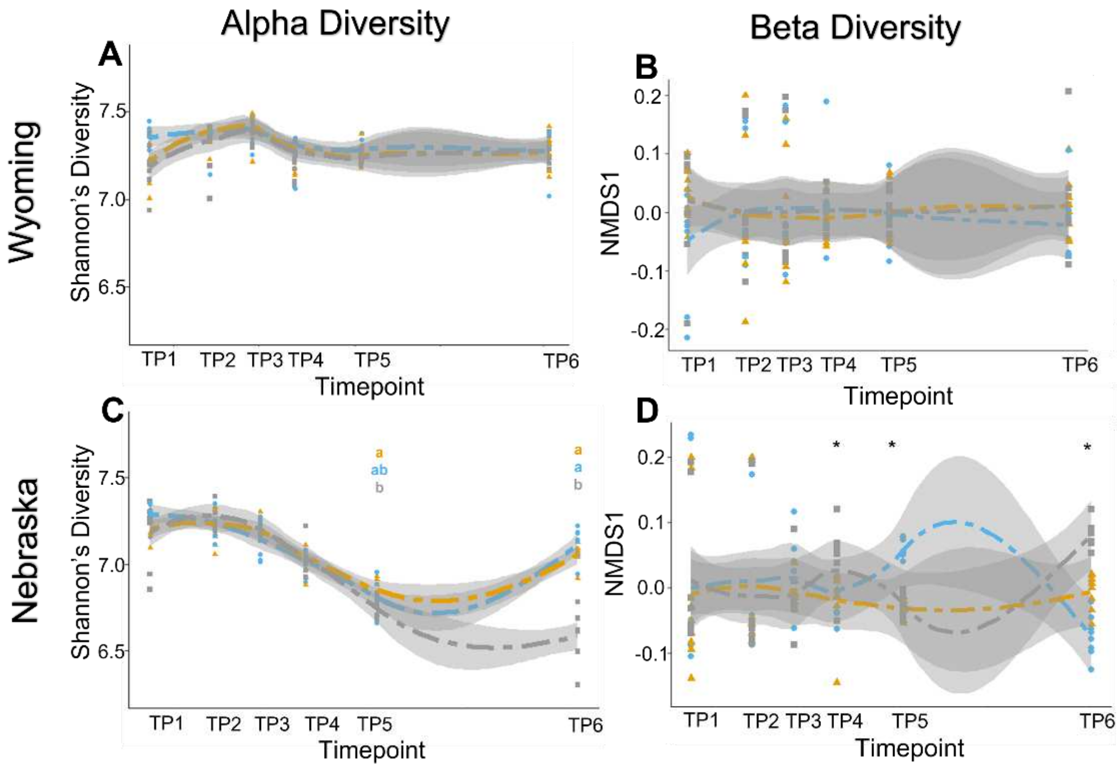

Figure 1.1. Glyphosate (blue), mixed selective herbicide (orange) and control (gray) treatments are represented as scatter plots with standard error shading. Temporal representation of the alpha diversity for the Lingle, WY site (A) and the North Platte, NE site (C) is based on Shannon's diversity metric. Letters a, b and ab correspond to color of treatment, and represent groups that are significantly different from each other based on Tukey's HSD test. Beta diversity is represented by Axis 1 of NMDS ordination plots constructed for each timepoint for the Lingle, WY site (B) and the North Platte, NE site (D). Significant differences between sample communities based on PERMANOVA are represented by an Asterix (*). A significance level of α = 0.05 was used for statistical analysis.

PERMANOVA analysis was used to determine the influence of treatments on the communities over six timepoints (Supplementary Figure 1.2). At the Lingle, WY plot, PERMANOVA analysis showed no significant differences between the treatments at any timepoint. In the North Platte, NE plot, we did not find significant differences during the first three timepoints, however we did observe significant differences in community composition between both herbicide and control treatments in the last three timepoints based on the PERMANOVA (Figure 1.1). Axis 2 of the NMDS plot can be visualized in Supplementary Figure 1.3. Like the alpha diversity results, we postulate that the

differences in community composition between the treatment plots and control plot are indirect and related to the influence of diversity of plant species present, rather than a direct impact on bacterial species (Weidenhamer & Callaway, 2010). The variation in microbial community diversity observed between these two sites also represents the effect of environment and soil conditions on the resiliency of the microbiome (Caggìa et al., 2023; Lupwayi et al., 2022).

Both plots had a similar relative abundance at the beginning of the season, however they diverged significantly throughout the season. Proteobacteria (25-30%) was the most dominant phyla in relative abundance, followed by Bacteroidetes (21-22%) and then Actinobacteria (11-13%) at both sites for all treatments the first timepoint (Supplemental Figure 1.4). While we observed temporal changes in microbial relative abundance throughout our sampling period, the treatments had little impact. We did not observe any depletion or enrichment of taxa for either treatment in the Wyoming plot over the sampling period as visualized in volcano plots (Supplementary Figure 1.5). In Nebraska, there was an overall depletion of Cyanobacteria in both the glyphosate and mixed selective treatment plots, however this was temporal and Cyanobacteria only accounts for 1.05% of the total relative abundance of species across the three treatments. In the glyphosate treatment, there was a depletion in a few OTUs of Proteobacteria (3 OTUs), Bacteriodetes (2 OTUs), Fibrobacteria (1 out) and Firmicutes (1 OTU), while in our mixed selective herbicide plot, we observed a depletion in Firmicutes (1 OTU) and an enrichment in Bacteriodetes (1 OTU). The vast majority of these OTUs were not significantly affected by the treatments, therefore we cannot attribute community differences to herbicide application.

While studies have shown differences in community composition when looking short-term or *in vitro* (Kepler et al., 2020; Boldt & Jacobsen, 1998; Rose et al., 2016), it's important to account for all variables that could be influencing the results. Short term or single timepoint field studies have also generated significant differences due to herbicide inputs (Arango et al., 2014). However, our study has demonstrated that the temporal and environmental influence are the greatest drivers in differences when looking at an entire growing season compared to herbicide treatment. Consistent with our study, long-term field studies examining the impact of herbicide inputs on the microbiome have observed few negative impacts on species richness and biomass (Cheng et al., 2008; Schlatter Daniel et al., 2017; Kepler et al., 2020; Aguiar et al., 2020; Weaver et al., 2007; Busse et al., 2001).

Function of bacterial communities are not impacted by herbicide treatments

Nutrient cycling by microbes can improve plant growth and resistance to disease, therefore it's essential to understand the impacts of herbicide application on the functional ability of the microbiome. Microbial extracellular enzyme production can be regulated by nutrient presence in the soil and a good indicator of nutrient cycling and soil health (Luo et al., 2017). Alpha glucosidase, beta glucosidase, beta-xylosidase and cellobiohydrolase contribute to carbon cycling and plant litter decomposition, which further promotes the growth and activity of soil bacteria (Adetunji et al., 2017; Štursová & Baldrian, 2011; Matsuzawa et al., 2016; Fan et al., 2012). Phosphatases mineralize organic phosphorous into a plant-available form (Nannipieri et al., 2011) and N-acteylglucosaminidase is involved in nitrogen cycling (Kang et al., 2005). In both plots, we did not observe any significant differences across the timepoints for enzyme activity,

however temporal changes throughout the season were observed. Our results are consistent with a study by Tyler, 2022, where only minor differences were observed in beta-glucosidase, cellobiohydrolate, N-acetylglycosaminidase and phosphatase enzyme activity over two years after application of glyphosate and 2,4-D (Tyler, 2022). Other studies have had variable, fluctuating results based on active ingredient and rate of application of herbicide (Lupwayi et al., 2022; Z. Du et al., 2018). Our results demonstrate that enzyme function is not affected by herbicide application, which can give insight into the impacts of weed management strategies on overall soil health (Figure 1.2).

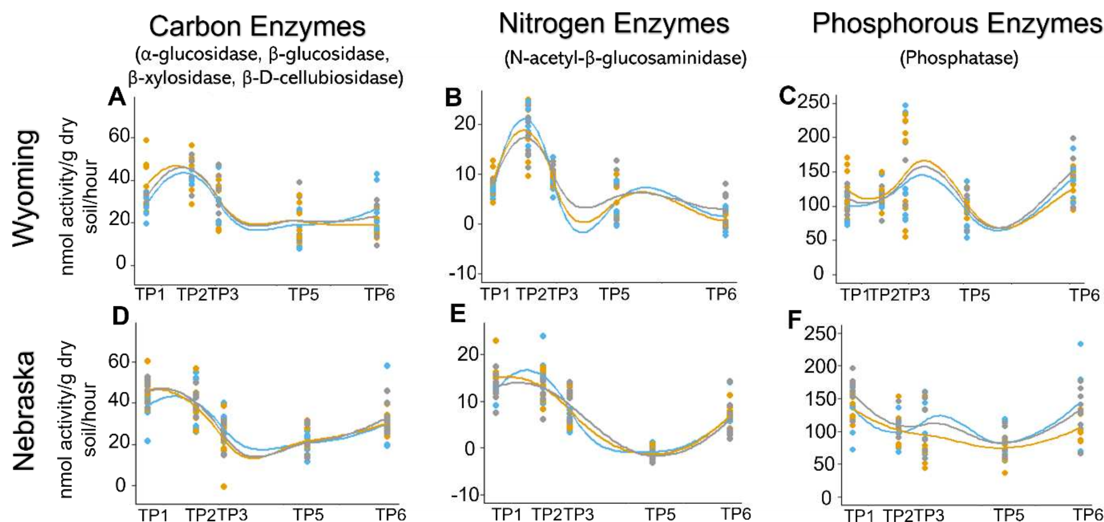

Figure 1.2. Glyphosate (blue), mixed selective herbicide (orange) and control (gray) treatments are represented. Extracellular enzyme production at five timepoints for the Wyoming (A-C) and Nebraska (D-F) sites and normalized to nmol/activity/g dry soil/hour. Carbon enzymes represent an average of α-glucosidase, β-glucosidase, β-xylosidase and cellobiohydrolase production, N-acteylglucosaminidase production represents relative nitrogen cycling and phosphatase represents phosphorous cycling. No significant differences are observed between the treatments throughout the timepoints.

At field application rates, soil respiration has been observed to be stimulated with the application of glyphosate (Lane et al., 2012; Means et al., 2007) or unchanged (Zabaloy & Gómez, 2008; Pereira et al., 2008), with limited studies seeing a reduction in microbial respiration. Decrease in microbial respiration could be plant-mediated due to less plant species present at treatment sites, rather than a direct effect of the herbicide (Rose et al., 2016). Our results did not show significant differences in microbial respiration between the glyphosate, mixed selective and control treatments (Supplementary Figure 1.6).

Functional gene abundance in the soil is another indicator of soil health. Overall, we did not find significant differences in functional gene abundance related to persistent herbicide treatment (Figure 1.3). We observed few differences in the first timepoint, however those changes disappeared later in the season. Ribosomal gene quantification for total bacteria found only one difference at the first timepoint in the Nebraska control plot. Within the nitrogen cycling genes, nifH, nirS, nirK and nosZ, only nifH showed significant differences between treatments at the first timepoint in the Nebraska glyphosate plot (Supplementary Figure 1.7). PhoC, a phosphorous cycling gene did not have significant differences between treatments or timepoints. Similarly, GH11 and GH18, functional genes involved in carbon cycling, did not see any significant difference between treatments or timepoints. Like extracellular enzyme production, previous studies involving functional gene presence are highly variable and dependent on experimental design. nifH was previously described to be negatively correlated to herbicide residue, while nirK and nosZ were positively, or not affected, however these results were compound dependent (Walder et al., 2022). From our exploration into

functional gene presence, there is minimal evidence to suggest that these herbicides are affecting soil function over a cropping season.

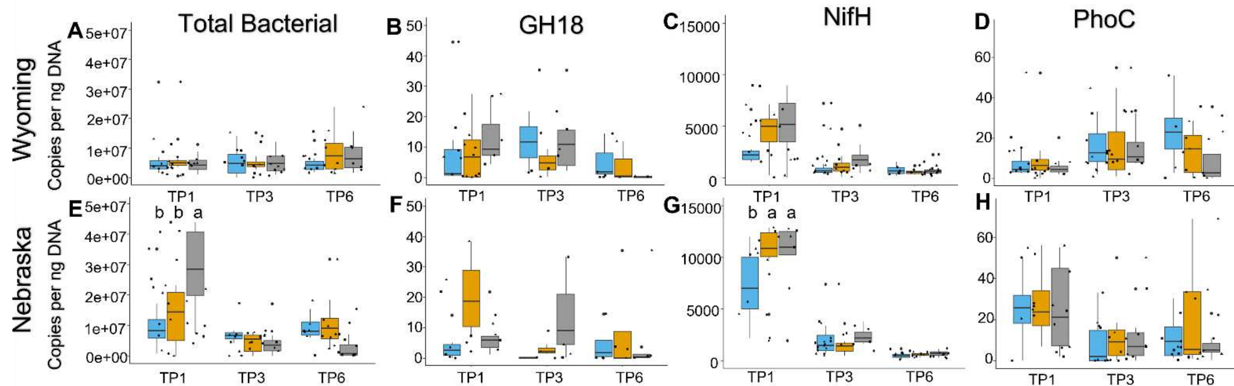

Figure 1.3. Glyphosate (blue), mixed selective herbicide (orange) and control (gray) treatments are represented. Qualitative PCR data demonstrating functional gene presence in soil samples for total bacterial genes (A,E), carbon cycling gene GH18 (B,F), nitrogen cycling gene NifH (C,G) and phosphorous cycling gene PhoC (D,H) for the Wyoming (A-D) and Nebraska (E-H) plots at the first, third and sixth sampling timepoints. Ct values from qPCR were normalized to a standard curve and expressed as copies per ng of DNA.

Like community composition, previous studies on the effect of herbicides on soil function have shown high variability and inconsistency in aspects such as herbicide rates, formulations, environmental or soil conditions (Nguyen et al., 2016). Studies observing changes in microbial function due to herbicide applications have been attributed the differences to environmental conditions (Means et al., 2007), soil organic matter (Haney et al., 2002; Tyler, 2022), soil moisture (Caggìa et al., 2023) or changes in plant-stimulated nutrient cycling (Damin et al., 2012) rather than direct impact of the herbicide itself. One study found that the influence of glyphosate and atrazine on nutrient cycling and mineralization was rate-dependent, however activity was stimulated at all rates, rather than hindered (Haney et al., 2002). Nutrient cycling can also be relative to dead plant litter on top of the soil surface after application that would be

absent in mechanical weeding practices, rather than due to direct impacts of the microbiome (Rose et al., 2016).

Glyphosate has a direct impact on bacterial strains *in vitro*

In contrast to the largely non-significant impact of herbicides on microbial structure and function under field conditions, our *in vitro* assay using individual bacterial isolates showed significant impact on the growth of most of the strains tested (Figure 1.4). Out of the 86 isolates used in the assay, 69 had significantly reduced growth when cultured in the presence of glyphosate, while 3 isolates had significantly increased growth. We did not observe any trends based on phylogeny on the species tested. Variability in growth within closely related species could be related to the incidence of glyphosate resistant genes. Bacteria have been classified into two clusters based on their enolpyruvylshikimate-3-phosphate synthase enzymes, class I that are sensitive to glyphosate and class II, insensitive to glyphosate, however classification can be variable even within species (Motta et al., 2018). Resistance to glyphosate can occur through the overproduction of EPSP synthase, degrading or detoxifying glyphosate, reducing uptake, or increasing export of glyphosate (Hertel et al., 2021). Bacteria that are sensitive to glyphosate, however, have been observed to survive when growing in amino acid rich media, as they are able to use the surrounding amino acids while their biosynthesis pathway is disrupted (Wicke et al, 2019). We observed strains that showed stimulated growth in the presence of glyphosate, which may be due to the bacteria using the herbicide as a nutrient source, such as phosphorous or carbon (Partoazar et al., 2011; Panettieri et al., 2013; Jacob et al., 1988; Lerbs et al., 1990). Since herbicides can act as a source of available nutrients while simultaneously being

potentially toxic to susceptible bacteria, these contrasting effects could lead to limited influences on the overall microbiome in the field (Lupwayi et al., 2022; Busse et al., 2001). The 10 mM rate used in this study is testing isolated strains exposed to a high rate of glyphosate. Under field conditions, the herbicide would be intercepted by plants during application and rapidly adsorbed to soil particles, decreasing the overall exposure to soil microbes (Duke & Powles, 2008). Previous *in vitro* studies have demonstrated high variation in laboratory and growth conditions (Wicke et al., 2019; Lone et al., 2014; Jeenie et al., 2011; Chávez-Ortiz et al., 2022), which could lead to misinterpretations of natural processes. Microbes living in natural or managed systems typically exist in communities of diverse organisms, rather than functioning alone as a single species (Singh et al., 2020). Our study indicates the importance of a diverse, cooperative microbiome in field soils that could improve resilience of the community, that cannot be achieved in a laboratory setting.

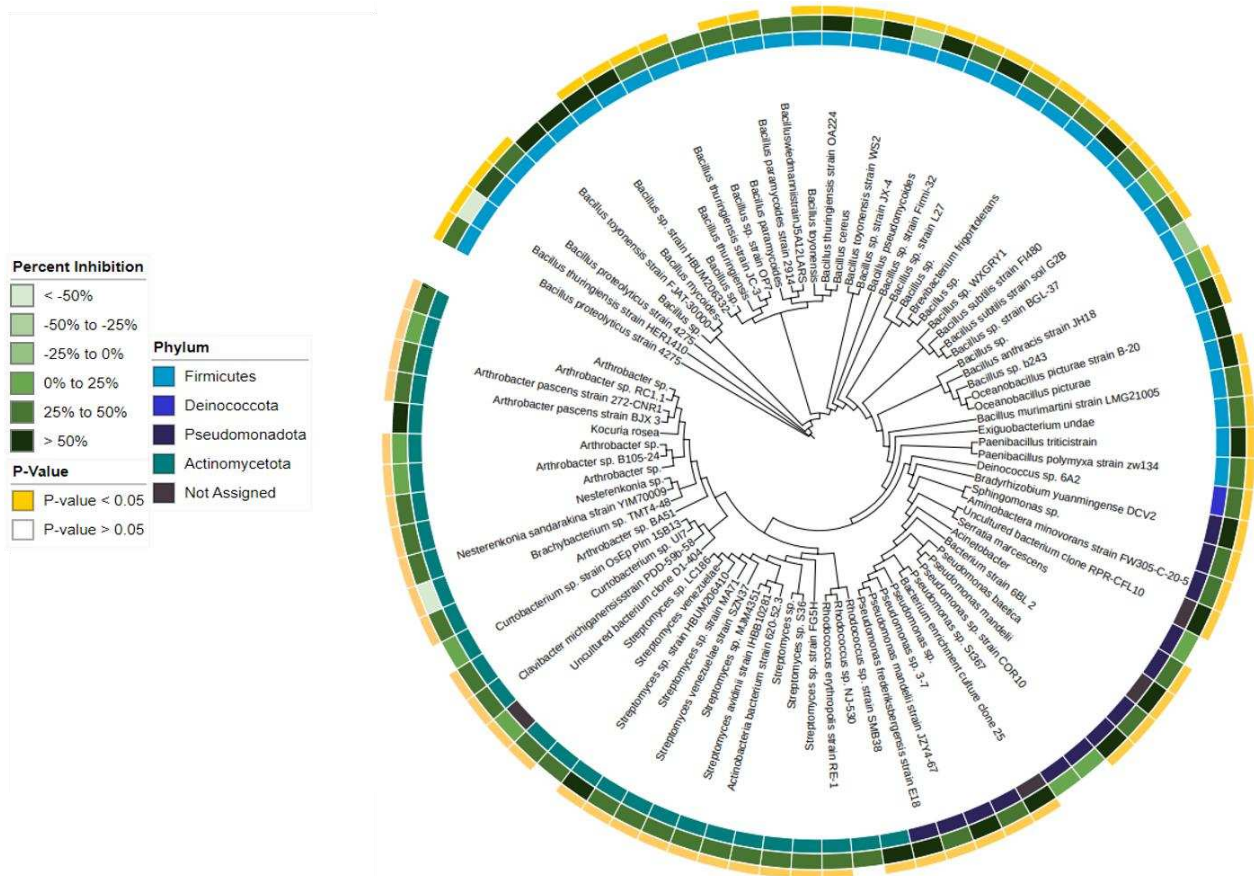

Figure 1.4. Phylogenetic tree of 86 bacteria isolated from soils representing phylum (inner circle), percent growth inhibition due to glyphosate (center circle) and significance (outer circle) based on a student's t-test and 0.05 significance level.

CONCLUSION

Information about the impact of herbicides on soil microbiome and assessing the toxicity of these compounds for soil microbial communities can give insight into required testingof herbicides during the regulatory process. . Previous studies examining the influence of herbicides on the soil microbiome have been highly variable both in experimental design and results. This study demonstrates a multi-site, well-replicated field study, that considers temporal variations in soil and plant activity throughout the

season. Bacterial community structure responded slightly due to herbicide treatment in just one of the two sites, however, these herbicides did not have an impact on nutrient cycling enzymes, functional gene abundance, or soil microbial respiration, which are important indicators of overall soil health (Dubey et al., 2019; Luo et al., 2017). Consequently, our results demonstrate that while certain bacterial strains may be impacted by direct herbicide exposure, microbial species interacting in communities under field conditions were relatively unchanged. Our findings provide evidence that repeated herbicide usage does not directly impact soil health and function.

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CHAPTER 2: DESIGN, CONSTRUCTION AND EVALUATION OF A BIOLOGICAL CONTROL AGENT FOR DISEASE CONTROL IN SUGAR BEET

INTRODUCTION

Soil-borne plant pathogenic fungi cause a variety of diseases, such as root, stem and crown rot, damping-off, and vascular wilts, resulting in significant economic losses in yield and quality of agricultural and horticultural crops worldwide (Jambhulkar et al., 2015; Mehta et al., 2014; Panth et al., 2020). Many of the most aggressive soil-borne fungal pathogens are predicted to increase globally by up to 3-fold by 2050 (Delgado-Baquerizo et al., 2020; Singh et al., 2023). Many soil-borne fungal pathogens are difficult to control by agricultural management practices or by application of chemical fungicides (Katan, 2017; You et al., 2020). In addition, the utilization of chemical fungicides is increasingly restricted due to the development of fungal resistance and negative impact on human, animal, and environmental health (Hu & Chen, 2021; Lucas et al., 2015; Shafique et al., 2016). Harnessing the beneficial potential of native microbes has emerged as one of the few logistically, eco-friendly, and economically feasible solutions for controlling various fungal disease (Graham & Strauss, 2021; Lahlali et al., 2022; Trivedi et al., 2021). However, the efficacy with the use of microbials for disease control can be highly variable (Mitter et al., 2019; Niu et al., 2020). Therefore, the primary question revolves around how microbiome mediated beneficial traits be successfully maintained in the field to increase resistance against fungal pathogens.

It is becoming increasing clear that potential microbial applications for plant protection depends on the understanding and manipulation of multispecies interactions that are critical for microbiota-mediated disease resistance (Asad, 2022; De Corato, 2020; Dimkić et al., 2022; Elnahal et al., 2022). Recent research suggests that combining species into biological control agents (BCAs) could confer efficient plant protection compared to individual strains and argue for the use of native locally adapted microbes for efficient plant protection in the field (Carrión et al., 2019; Mazzola & Freilich, 2017; Sarma et al., 2015). BCAs can constitute different microbes with a combination of mechanisms to inhibit pathogen growth, promote plant health and improve disease resistance (Domenech et al., 2006; Kaur et al., 2022; Kavino et al., 2016; Niu et al., 2020; Solanki et al., 2019). Compared to the application of individual species, combining synergistic, functionally diverse strains in a consortium could improve efficacy of the community and provide greater benefits to agricultural crops (Hays et al., 2015; Niu et al., 2017). Furthermore, application of multiple strains may be more adaptable to changing environmental conditions (Ruano-Rosa et al., 2014). Designing and assessing a BCA is not an easy task, and present research lacks a workflow to streamline the process and predict the success of the organisms under field conditions. Numerous factors must be considered, particularly the ability of individual biological control agent to inhibit pathogen and promote plant growth, their compatibility to each other, and their complex interactions within consortia and within the rhizosphere environment (Liu et al., 2019; Mazzola & Freilich, 2017; Sarma et al., 2015; Thomloudi et al., 2019).

Sugar beet is an important crop that accounts for about 30% of the worldwide sugar production and it is being studied as a major producer of ethanol (Dohm et al., 2014; Maung & Gustafson, 2011). Sugar beet management involves disease control for a variety of pathogens. Diseases such as Rhizoctonia, Fusarium, Aphanomyces, and Pythium, viruses such as beet curly top virus and rhizomania, and beet cyst nematode, can affect root sucrose quantity and quality, causing major issues for production (Biancardi et al., 2010). Root rot pathogens are of particular importance because profit from sugar beet production is dependent on the yield and quality of the sucrose in the roots (Jacobsen, 2006). *Rhizoctonia solani* AG2-2 IIIB can cause seedling damping off and mature root and crown rot, affecting more than 24% of sugar beet acreage across the United States (Jacobsen, 2006). *R. solani* AG2-2 IIIB can also form sclerotia, allowing the pathogen to remain in the soil until environmental conditions are favorable (Haque & Parvin, 2021). A few sugar beet associated plant growth-promoting bacteria have been isolated and tested for their in planta activity to induce immune response and disease suppression (Carrión et al., 2019; Karimi et al., 2016; Kiewnick et al., 2001).However, by adopting a systems-based approach to formulate novel consortia, we can effectively harness and characterize the beneficial potential of native microbial strains through laboratory and greenhouse assays.

The goal of this study is to design, formulate and apply biological control agents consisting of bacterial strains initially isolated from sugar beet producing agroecosystems and possessing beneficial traits to antagonize fungal pathogens. We propose that a mixture of multiple antagonistic and compatible bacterial strains will result in better control of fungal pathogens, compared to individual strains. Our

systematic approach begins with isolating bacteria from sugar beet agroecosystems, characterizing bacterial isolates by sequencing the 16S rRNA gene, and screening for antagonistic activities against three fungal pathogens (*Rhizoctonia solani, Fusarium oxysporum,* and *Fusarium secorum*) by performing competition assays *in vitro*. We then formulated multiple strain biological control agents with different complexities and explored the efficacy of these communities in controlling *Rhizoctonia solani* AG2-2 IIIB. Using the most effective BCAs, we performed *in planta* experiments to quantify success in controlling damping off and mature rot symptoms caused by *Rhizoctonia solani* AG2-2 IIIB in a susceptible sugar beet variety. Our study demonstrates the importance of understanding interspecies interactions when developing BCAs and promotes further exploration into microbial alternatives to control economically important soil pathogens.

MATERIALS AND METHODS

Extraction and storage

For our study we utilized a culture collection of 136 bacterial strains initially isolated from sugar beet producing agroecosystems. Soil samples were serially diluted on nutrient agar (NA) plates and incubated for 3-5 days at 28C. Bacterial colonies that showed differences in the morphology, color, and growth characteristics were picked and streaked on to individual NA plates and incubated for 3-5 days at 28C. To ensure purity, two further rounds of successive streaking on fresh NA plates were performed from the individual NA plates. DNA was extracted from individual isolates using the DNEasy Ultra Clean Microbial Kit (QIAGEN, Hilden, Germany) and Sanger sequencing

using primers 27F and 1492R was performed at Eurofins Genomics USA for strain level identification. Isolated strains were stored in glycerol in -80C freezer until use.

Fungal pathogens, RZC24 R-1 (*Rhizoctonia solani* AG2-2 IIIB), 102-6a (*Fusarium oxysporum)* and FOB220a (*Fusarium secorum)* were kindly provided by Dr. Kim Webb, USDA-ARS (Sugar Beet Research Unit, Fort Collins, CO). The fungal isolates were initially isolated from sugar beet plants (Webb et al., 2011). The fungal isolates were grown in potato dextrose media and stored in glycerol in -80C.

Screening of bacterial strains antagonistic to fungal pathogens

Preliminary Screening

A total of 136 bacteria strains and 3 fungal pathogens were cultured on NA plates from their glycerol stock solutions. The plates were incubated for 3-5 days at 28C. From these plates a second round of streaking and incubation was performed to verify the colony purity. For antagonistic assays we placed 7 mm agar plugs of 1 week old fungal cultures in the center of a petri dish on ½ strength potato dextrose agar (PDA). A 20 mm streak of four different bacteria was inoculated 20 mm away from the center of the agar plug (Supplementary Figure 2.1). After 6 d, hyphal growth inhibition in the direction of the bacterial strain was noted on the plates. The procedure was repeated twice.

Secondary Screening

From our preliminary screening, we selected thirty-two bacterial strains that showed clear sign of antagonism for at least one fungal pathogen. For the secondary screening, the same approach was used as described in the previous section, however

a single bacterial strain was streaked onto each plate. Three replicates were conducted for each bacterial-fungal combination. Control plates for each fungus consisted of individual strains growing on 4 plates without bacteria. After 8 d, percent inhibition was calculated by comparing the growth of the treated plates to control plates (Figure 2.1).

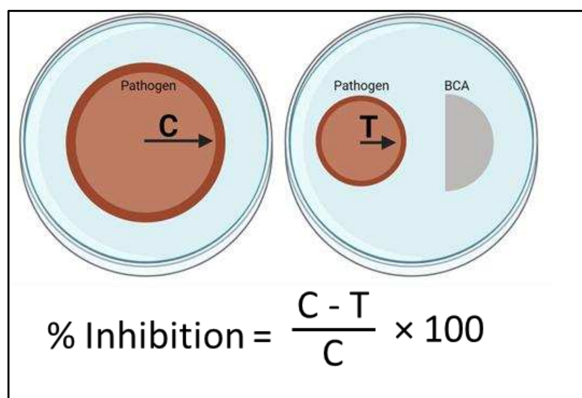

Figure 2.1. Percent inhibition calculation on dual culture assay plates.

Sanger sequencing

Strains screened after secondary were subjected to another round of Sanger sequencing to verify the strain level identity. Selected bacterial strains were cultured on NA and incubated for 5 days at 28C. Colonies from the bacterial plates were picked, grown in nutrient broth and incubated for 24 h at 150 rpm and 25C. DNA was extracted using the UltraClean Microbial Kit (QIAGEN, Hilden, Germany). PCR was performed with 4 uL of DNA and MyTaq Red Mix (Meridian Bioscience, Cincinnati, OH, USA) on the Bio-Rad thermocycler (Bio-Rad, Hercules, CA, USA). 27F/1492R primers were used to target the 16S rRNA region of the bacterial samples. We also verified the strain level identity of fungal pathogens by using the similar methods except for primers ITS1F/ITS4 were used to target the ITS region. Amplified DNA was quantified on an electrophoresis

gel followed by an ExoSap-IT (Thermo Fisher Scientific, Waltham, Massachusetts, USA) cleanup remove excess primers. Samples were sent to Eurofins Genomics USA for sequencing.

Bacterial-bacterial competition assays

Thirty-two bacterial isolates showing the highest percent growth inhibition towards *Rhizoctonia solani* were cultured on NA. Pure bacterial samples were added to 1x PBS and vortexed well. A bacterial lawn of 100 ul of a single strain was spread on a nutrient agar plate. Immediately after the lawn was dried, 10 ul of each of the other 31 bacteria were equally spaced in a grid on the plate. Plates were incubated for 48 hours. Inhibition zones, or halos, around the outside of the pipetted bacteria were identified.

BCA formulation and BCA-fungal interaction assay

BCA formulation: Pure bacterial colonies were cultured in 1.5 ml nutrient broth (NB) for 7 d in 2 ml tubes at 25C and 100 rpm. Cultures were pelletized through centrifugation at 4,000 rpm for 20 minutes. The bacterial cells were washed twice with 1x PBS. The supernatant was removed, replaced with 1.5 ml of fresh NB and adjusted to 1×10^8 - 3×10^8. Equal amounts of each bacteria, depending on the number of species included in the community, were combined into 1.5 ml microcentrifuge tubes and vortexed for 5 seconds immediately before adding the community into the 96-well assay plate. 90 communities were constructed with different combinations of 1-10 bacterial isolates from preliminary dual culture assays.

BCA fungal interaction: Two-week-old *Rhizoctonia solani* AG2-2 IIIB cultures were grown on ½ strength soft PDA without supplemental agar. After 5 d of growth, ten mycelial plugs were collected from the outside of the growth region and added to a 1.5 ml microcentrifuge tube. Plugs were ground with a pellet pestle until smooth. NB was added to the tube and vortexed to mix well. The mycelial mixture was transferred to a sterilized blender. The microcentrifuge tube and pipet were rinsed with NB twice to remove all fungal cells. 50 total agar plugs were added to 100 ml of NB in the blender and blended until completely liquid. Fungal suspension was filtered through a cheese cloth. 750 ul of pure NB was added to each well of a 2 ml 96-well plate. 200 ul of fungal suspension was added to each well with 50 ul of bacterial communities in each treatment well. The plate set up was randomized and four replications were completed for each community. Four positive control replicates with 800 ul total of NB and 200 ul of fungal suspension were included on each 96-well plate. Four negative control wells contained only NB. Plates were incubated for 7 d at 25C and 150 rpm. This process was completed twice, first with the 90 original BCAs and a second time using the 45 BCAs showing the highest growth inhibition of the pathogen.

DNA extraction and qPCR

DNA was extracted using the MagAttract Microbial DNA Kit (QIAGEN, Hilden, Germany) on the Kingfisher Duo (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Quantitative PCR was performed on the Bio-Rad thermocycler (Bio-Rad, Hercules, CA, USA) with a *Rhizoctonia solani* AG2-2 specific primer (Supplementary Figure 2.2). Fungal gene abundance in wells treated with bacterial inoculant were

compared to fungal gene abundance in control wells without bacterial inoculants and percent inhibition was calculated.

Serial dilution

Due to the concern of *Serratia marcescens* 10A.1A.P216 being a competitor of the other two isolates based on bacterial-bacterial competition assays, we performed a serial dilution of C53, a consortia of *Brevibacillus* sp. P2.F4.A, *Bacillus cereus* 4B.1B.PK and *Serratia marcescens* 10A.1A.P216, to confirm that all three species would be compatible over an extended period of time. The three individual strains were cultured separately in NB for 3 d. The cultures were centrifuged and washed twice in 0.03 $MgSO_4$ before being resuspended in NB. The cultures were adjusted to $1x10^8$ cells and combined equally together. C53 culture was incubated at 25C and 150 rpm for 7 d. A serial dilution was performed with 0.03 $MgSO_4$ and 10^{-5} dilution was spread on a nutrient agar plate. Colonies were counted and identified after 3 d.

In planta assay to assess the efficacy of selected BCAs to control Rhizoctonia Solani AG2-2 IIIB

Preparation of fungal inoculum

The workflow of the in-planta experiment is described in Figure 2.2. Barley, which is highly susceptible to *R. solani*, was used to prepare the fungal inoculant. Barley grains were soaked in distilled water to about 1 cm above the top of the barley. After soaking overnight, the water was poured out until just below the barley surface, autoclaved, then cooled overnight. The barley was broken into individual seeds and 7

mm plugs from the outside edge of a 7 d old *R. solani* culture were added to the barley every 2-3 cm and covered with grains. The barley was incubated at 25C for 2 weeks. After 2 weeks, the barley was broken up into individual grains and allowed to dry under a laminar hood. The grains were then added to a sterilized blender and pulse ground until about ¼ of the original size. Barley grains were sieved through a 2.36 mm sieve and separated into 2.5 mg aliquots. Additional 2.5 mg aliquots of sterilized, pulse-ground barley were used for the control pots.

Biological control agent preparation

We constructed three BCAs based on their efficacy to inhibit fungal growth in the in vitro assays. Individual bacterial strains were added to 35 ml of NB and incubated at 30C and 100 rpm for 48 h. Bacteria were washed three times by centrifugation for 10 min at 4,000 g, supernatant was removed and the remaining bacteria was resuspended in sterilized 0.03M $MgSO_4$. *Serratia marcescens* 10A.1A.P216, *Bacillus subtilis* 1A.1B.P216, *Brevibacillus* sp. P2.F4.A and *Bacillus cereus* 4B.1B.PK were adjusted to 1×10^8 - 3×10^8 cells. C1: *Serratia marcescens* and C15: *Bacillus subtilis* were separated into to 2 aliquots of 450 ml of 0.03M $MgSO_4$. Strains in C53: *Serratia marcescens* 10A.1A.P216, *Brevibacillus* P2.F4.A and *Bacillus cereus* 4B.1B.PK, were separated into 2 aliquots of 150 ml and combined immediately before seed soak or drench.

In planta damping off experiment

Sterilized potting mix and greens grade sand were combined at a 50:50 ratio in 4.5” pots. 2.5 g of sieved fungal barley inoculant or sterilized barley was incorporated

into the pots 4 d before sowing. Pots were watered to holding capacity. *Rhizoctonia solani* susceptible sugar beet seeds, variety 4012RR (DLF, Longmont, CO, USA) were surface sterilized by soaking in 70% ethyl alcohol for 2 minutes, followed by a rinse with DI water. Seeds were then transferred to a 5% hydrogen peroxide solution for 3 minutes, washed 10x with DI water, and dried in a laminar flow cabinet for 20 minutes. Seeds were soaked in one aliquot of community suspension for 2 h. Immediately after soaking, seeds were sown 5 mm deep using sterilized forceps. 10 seeds were sown per 4.5" pot. 20 ml of the second aliquot of bacterial suspension was drenched on each corresponding pot. Positive controls contained bacterial suspension without fungal inoculation. Negative control seeds were soaked in a sterilized 0.03 M $MgSO_4$, followed by a 0.03 M $MgSO_4$ drench. Each treatment had eight replicates and the pots followed a complete randomized design to account for variation within the greenhouse (Supplementary Figure 2.3). Pots were watered to 60% of the water holding capacity every 3 d. Plants were fertilized with $1/4^{th}$ tablespoon Osmocote 14-14-14 fertilizer 7 days post sow (DPS). Seedlings were thinned to 6 plants per pot after approximately 7 DPS. Soil samples were collected from the first 3 reps of each treatment immediately before seeding. Survival and germination rates were recorded every 3 d. At 28 DPS, rhizosphere soil from the seedlings was collected. Roots were separated from the shoots and washed to remove rhizosphere soil. Both roots and shoots were transferred to a freezer for further analysis. Roots and shoots from replicates 6, 7 and 8 were flash frozen to be used for transcriptomic analysis in a future study.

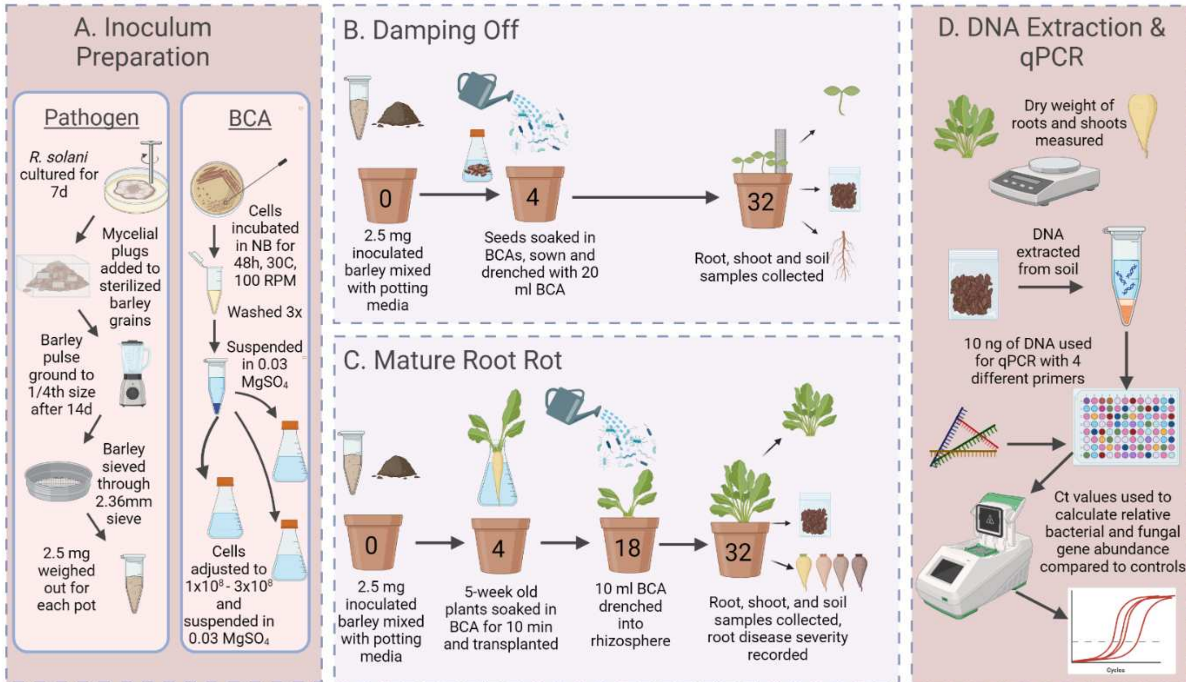

Figure 2.2. Representation of the experimental set up for the *in planta* experiments, starting with the inoculum preparation of both the fungi and the bacterial biological control agent (A), the seedling damping off experiment (B), the mature root and crown rot experiment (C) and laboratory analysis to identify the to quantify gene abundance of pathogen and bacteria in the soil (D).

In planta root and crown rot experiment

Seeds were sterilized as described above. 5 seeds were sown into sterilized 50:50 potting mix:greens grade sand in 2” pots. After 10 d, seedlings were thinned to 1 per pot. Seedlings were fertilized with 1/8th tablespoon of Osmocote 14-14-14 fertilizer and watered thoroughly for 35 d. Fungal and control inoculants were made as described above and mixed into 4.5” pots with sterilized 50:50 potting mix:greens grade 4 d before transplanting. Bacterial communities were cultured and suspended in 0.03 $MgSO_4$ as described above. 5-week-old sugar beet plants were removed from 2” pots and roots were washed to remove the rhizosphere soil. Roots were then soaked in 450 ml aliquot of bacterial 0.03 M $MgSO_4$ solution for 10 min and transplanted into a 4.5” pot with or

without fungal inoculant. Eight replicates were completed for each treatment and pots followed a complete randomized design to account for variation within the greenhouse (Supplementary Figure 2.4). Pots were placed 4" apart to avoid competition between pots. A second drench of 10 ml of bacterial MgSO4 suspension was completed 14 days post-transplant (DPT). Pots were watered to 60% of the water holding capacity of the pots every 2 d and plants were fertilized 7 DPT with 1/4th tbsp of osmocote 14-14-14 fertilizer. Number of leaves and height of stem were recorded at the time of transplanting. Percent crown rot was calculated every 2 d by recording the dead leaves due to *R. solani* AG2-2 and comparing to the total emerged leaves. 28 DPT the rhizosphere soil was collected. Roots were washed and given a disease score from 0-7 based on greenhouse protocols described by the USDA-ARS Sugar Beet Research Unit (Fort Collins, CO, USA) (Supplementary Figure 2.4). Roots and shoots were then transferred to a freezer for further analysis. Roots and shoots from replicates 6, 7 and 8 were flash frozen for transcriptomic analysis in a future study.

Dry biomass

Root samples were washed again to remove any remaining soil. Root and shoot samples were put into a paper envelope and placed in a drying oven for 72 hours at 60C. After 72 h, samples were weighed and dry weight was recorded.

Soil DNA extraction and Quantitative PCR

Frozen soil samples were thawed. About 250-300 mg of soil was added to a bead tube and DNA was extracted using the DNEasy PowerSoil Pro Kit (QIAGEN, Hilden, Germany). DNA concentration was determined using a Quant-IT PicoGreen

Assay (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and a Fluorskan plate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA). DNA quality was assessed using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). DNA concentration was normalized to 10 ng/ul for all samples and qPCR was conducted using 2 ul of DNA on the Bio-Rad thermocycler (Bio-Rad, Hercules, CA, USA). Individual qPCR runs were completed with primers for *Rhizoctonia solani* AG2-2, total bacteria, gammaproteobacteria and Firmicutes (Supplementary Figure 2.2).

Statistical Analysis

The visualization of the percent growth inhibition and compatibility assays were performed using the interactive tree of life (iTOL) interface (Letunic & Bork, 2021). The bacterial phylogenetic tree was constructed using Clustal Omega by EMBL-EBI (Madeira et al., 2022). The tree and the metadata files derived from the dual culture and compatibility assays were imported into iTOL interface. The dual culture assay data is displayed as percent inhibition, and the bacterial compatibility assay data is displayed as a 0 (not compatible) or a 1 (compatible).

The Shapiro-Wilkes test was used to confirm normality of all datasets. Normally distributed data was analyzed using a two-way ANOVA to identify significance of the bacterial community and pathogen treatment. Non-normal data was analyzed using a Kruskal-Wallis non-parametric test for comparing the influence of four bacterial communities, and a Wilcoxon rank-sum non-parametric test was used to determine significance of the pathogen inoculation. A significance level of 0.05 was used for all statistical analyses. All statistical analyses were completed using R v 4.0.5 (R Core

Team & Team, 2022). Boxplots and scatter plots were made using ‘ggplot2’ (Wickham, 2016).

RESULTS AND DISCUSSION

Dual culture assays

Our results demonstrate that a wide diversity of both gram-negative and gram-positive bacteria can have antagonistic effects on soil fungal pathogens. Our results are in line with the conclusions from Duran et al. (2018) who suggested that the biocontrol activity of plant associated bacteria is a redundant trait and is essential for plant survival. BCAs can promote plant health through priming of plant immune responses or target the pathogen directly through production of secondary metabolites, such as lipopeptides, biosurfactants, bacteriocins, volatiles and hydrolytic enzymes, competition for resources or predation (Gupta & Bar, 2020; Helfrich et al., 2018; Nishad et al., 2020; Parratt & Laine, 2018; Schmidt et al., 2017; Zaid et al., 2022; Zelezniak et al., 2015). Dual culture assays indicate high diversity in mechanisms of action against the three pathogens (Figure 2.3A). The presence of an inhibition zone between the two species indicates the production of an antimicrobial metabolites (Figure 2.3A-A). We also observed direct interaction between the bacteria and pathogen, indicating bacterial mycophagy (Figure 2.3A-B) and the formation of biofilms (Figure 2.3A-C).

Out of the 136 bacteria tested, (15%) showed high inhibition rates to the three fungal pathogens (Figure 2.3B). These included 14 gram positive species from the phylum Firmicutes (including genus *Bacillus* (11) and *Brevibacillus)* and Actinobacteria (genus *Curtobacterium* and *Streptomyces*)), as well as 7 gram negative species from

the phylum Proteobacteria (including those belonging to genus *Acinetobacter, Pantoea, Serratia* and *Pseudomonas* (4)). There are several reports that have described the antagonistic activities of the bacterial genus screened in this study against a range of fungal pathogens (Garbeva et al., 2004; Gkarmiri et al., 2015; Jamali et al., 2020; Mavrodi et al., 2012; Shah et al., 2023; Solanki et al., 2015). Although all the selected strains have high antagonistic activity against the pathogens (31.5% against *R. solani,* 28.7% against *F. oxysporum,* 23.2% against *F. secorum)*, strain P4.A7A showed the highest inhibition compared to the other strains. Sanger sequencing results indicate that this species is most closely related to *Bacillus velezensis.* The majority (52%) of bacterial strains found to inhibit pathogen growth in vitro were belonged to genus *Bacillus*. This genus is commonly used as BCAs, due to their ability to form endospores and colonize the host plant. Endospores can remain in the environment for extended periods of time and withstand extreme environmental conditions, such as desiccation and heat (Melnick et al., 2008; Nicholson, 2002). *Bacillus* BCAs have been studied to be highly effective against sugar beet pathogens, such as Cercospora, Pythium and Fusarium (Jacobsen et al., 2004). This genus has been studied to have many mechanisms of action against *Rhizoctonia,* particularly due to the production of antifungal compounds such as lipopeptides and volatiles (Moyne et al., 2004; Romero et al., 2007; Zhu et al., 2023).

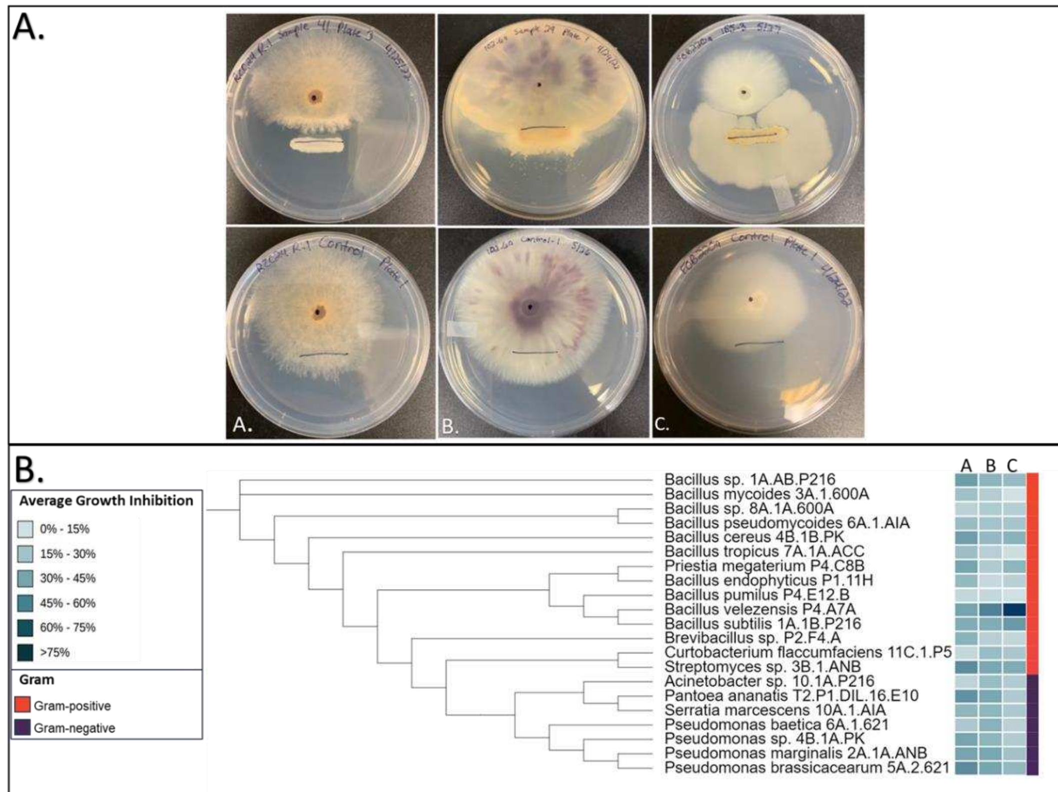

Figure 2.3A. Dual culture assay plates showing the diversity in antagonistic mechanisms of bacterial isolates against fungal pathogens. A) *Rhizoctonia solani* AG2-2 IIIB: *Bacillus cereus* 4B.1B.PK (B) *Fusarium oxysporum: Pseudomonas brassicacearum* 5A.2.621 (C) *Fusarium secorum: Bacillus velezensis* P4.A7A Figure 2.3B. Heat map demonstrating the average fungal growth inhibition in the 21 gram positive and gram negative bacteria tested in the secondary dual culture assays.

Bacterial compatibility assays

Successful consortium, in which a disease is effectively controlled, is rare, and in-depth research is needed to understand the best way to optimize these communities for tasks such as biocontrol (Faust, 2019; Prigigallo et al., 2022). While BCAs with multiple strains have been studied to have increased control of organisms (Carrión et al., 2019), some studies have also discovered that there is no significant benefit to including multiple biocontrol strains compared to individual strains (Georgakopoulos et al., 2002; Stockwell et al., 2011; Vanneste & Yu, 1995; Yin et al., 2022). Understanding the ability

of a potential bacterial strain to suppress pathogen growth is important when designing a BCA, however equally as important is understanding their compatibility with other strains. It is possible that, if two strains are competing with one another, they may not be able to reach a certain population threshold that is conducive to the production of antimicrobial metabolites (Haas & Défago, 2005). To test the compatibility of bacterial strains, we used a plate-based method in which we grew the different bacterial strains in a lawn of single bacteria. Halos present around inoculated bacteria on competition assay plates indicated an inhibition zone and therefore an antagonistic relationship between species (Figure 2.4A). The results of our compatibility assay showed variation in competition among strains (Figure 2.4B). To draw conclusions from the compatibility assays, it is important to identify which strains comprised lawn, and which were pipetted into the lawn. If a strain was applied onto the bacterial lawn and a halo is present, this indicates that the applied strain is more competitive than the lawn strain. Alternatively, a robust and competitive lawn strain would show fewer competitors. For example, the *Pseudomonas baetica* 6A.1.621 lawn (lawn 18 in Figure 2.4B) was inhibited by 48% bacterial strains that were applied on top of it. Therefore, this bacterial strain is less competitive. Alternatively, *Serratia marcescens* 10A.1.AIA (lawn 17 in Figure 4B) had the highest rate of competition with other species as we observed halos on 59% of the bacterial lawns tested (horizontal boxes), however none of the strains produced halos when *Serratia* was the lawn (vertical boxes), indicating that this *Serratia* strain is more robust. *Serratia marcescens* has been observed to employ a Type VI secretion system to inject antimicrobial toxins into other bacterial species, enhancing it's competitive and opportunistic lifestyle (Alcoforado Diniz & Coulthurst, 2015; Murdoch et al., 2011). The

observed competitive interactions highlight the varying degrees of competitiveness among these bacterial strains.

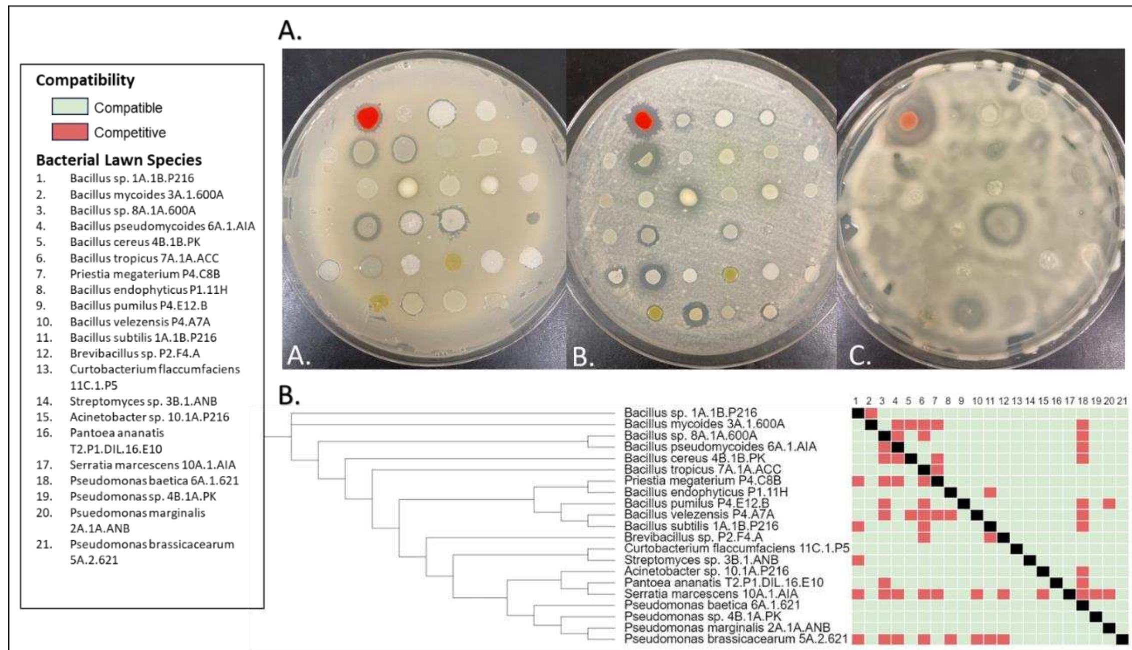

Figure 2.4A. Bacterial compatibility assay on nutrient agar plates. Halos around bacterial cultures indicate competition between two species. Figure 2.4B. Bacterial-bacterial competition assay results to assess compatibility between strains. Vertical boxes indicate bacterial lawns applied to the agar plates, while horizontal boxes represent the strains that were pipetted onto the lawn. Green boxes represent compatibility between species (no halos present) and red boxes represent competition (halo present).

The complexity of species interactions when designing BCAs with diverse species emphasizes the need for careful consideration and testing. Two strains may be compatible in a nutrient-rich, pathogen-free environment, however mechanistic compatibility is also an important aspect to consider. For example, one study involving fireblight observed the production of an extracellular protease by *Pseudomonas* negatively impacting the biological control ability of two strains of *Pantoea* (Stockwell et al., 2011). Alternatively, competition between two strains could potentially lead to the induction of antimicrobial compound production (Maurhofer et al., 2004), or the

pathogen could be triggered to express pathogenesis-related genes that would not be expressed when competing with a single strain (Stockwell et al., 2011). To increase the complexity of these interactions, certain strains may occupy different niches in the soil environment and therefore be incompatible in vitro, however in a natural setting these organisms would not necessarily interact (Pliego et al., 2008; Thomloudi et al., 2019). While in vitro assays are a crucial step in understanding the potential efficacy of BCAs, further testing is required to fully comprehend the interspecies interactions within a consortia.

Competition assays to assess ability of strains or consortia to inhibit *R. solani*

To better understand the potential benefits of individual or combined BCAs, we evaluated their ability to antagonize the fungal pathogen through percent fungal growth inhibition when compared to positive controls that contain the same amount of starting fungal inoculum. The preliminary in vitro screen of all 90 bacterial communities in NB showed a wide range of success in reducing pathogen growth. We did not find any specific trends based on the number of bacterial strains included in the communities (Figure 2.5A), indicating that the influence of strain has more of an impact than the number of species included in the community. For example, the single bacterial communities showed similar average inhibition (91.6%) against fungal pathogen when compared BCAs consisting of 8 (95.0%), 9 (94.2%) or 10 (16.4%) species.

The 45 biological control strains or consortia with the highest growth inhibition were selected for a secondary screen with the *Rhizoctonia solani* AG2-2 specific primer. The secondary screen showed high rates of growth inhibition compared to control wells.

Like the preliminary screen, we did not see any trends based on the number of bacterial strains included in each community or based on phylogeny (Figure 2.5B), and we observed a high rate of variation between replicates in the 96-well plate competition assays.

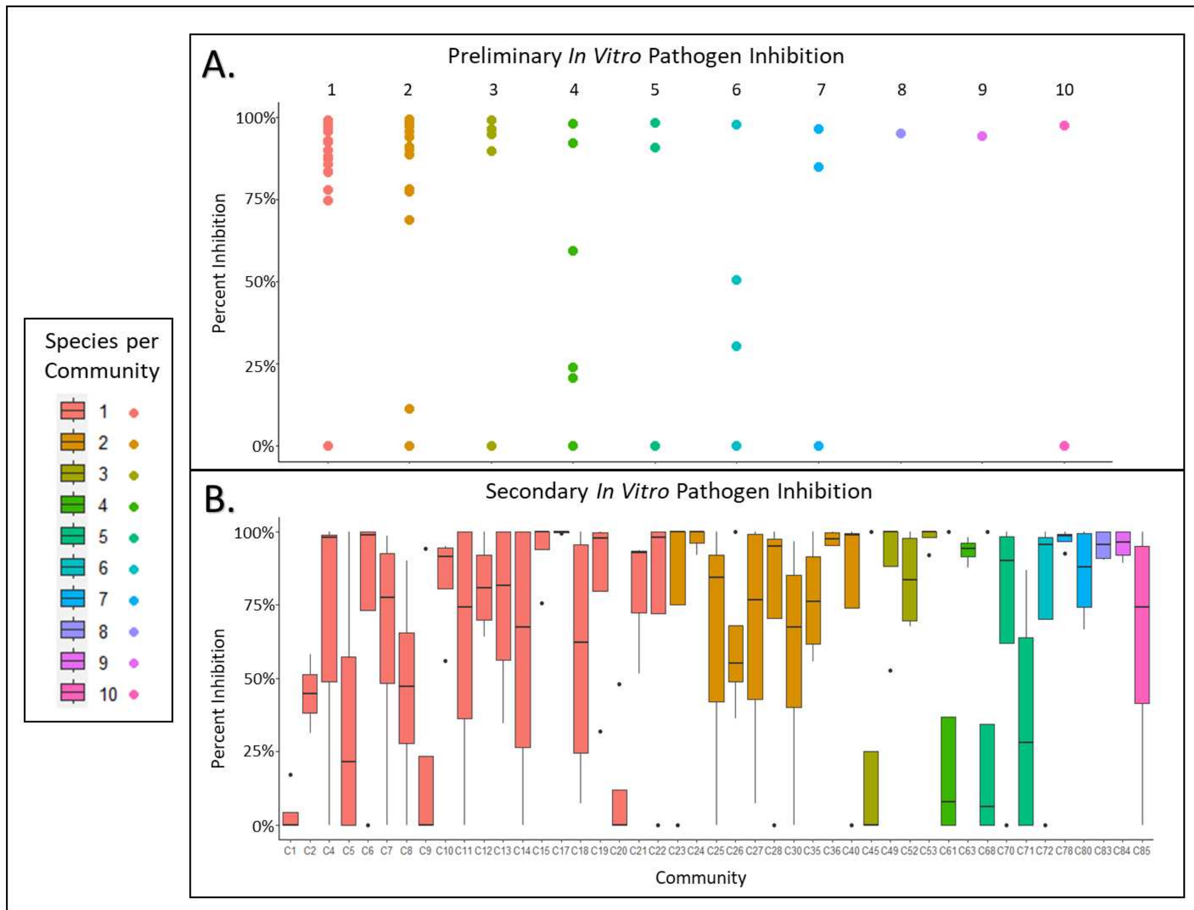

Figure 2.5. Percent inhibition based on 96-well plate qPCR data with a *Rhizoctonia solani* AG2-2 specific primer compared to control wells without bacterial inoculation. (A) Represents the initial 90 communities tested, grouped by the number of species per community. (B) Represents the 45 communities tested in the secondary in vitro screening. The colors coincide with the number of species per community as in Figure A.

The communities with the highest pathogen growth inhibition in our 96-well plate competition assay were C15 and C53. C15 is a single species of *Bacillus subtilis* 1A.1B.P216. *Bacillus subtilis* has been extensively studied for its control of *Rhizoctonia solani* due to its ability to produce antimicrobial compounds such as iturin A and surfactin (Al-Fadhal et al., 2019; Asaka & Shoda, 1996; Khedher et al., 2015). C53 was made up of three bacterial strains: *Bacillus cereus* 4B.1B.PK, *Brevibacillus* sp. P2.F4.A, and *Serratia marcescens* 10A.1A.P216. *Bacillus cereus* has also been observed to inhibit plant pathogens in vitro (Elkahoui et al., 2012; Handelsman et al., 1990) and in the field (Zhang et al., 2016). Members of *Brevibacillus* have previously been studied to inhibit growth of many soil pathogens (Ahmed, 2017; Chen et al., 2017), and both *Brevibacillus* sp. and *Serratia marcescens* can produce chitinase, which can degrade fungal cells walls and induce defense responses in plants (Masri et al., 2021; Someya et al., 2004). While *Serratia marcescens* 10A.1A.P216 demonstrated high rates of competition with the other bacterial species in the compatibility assays, it's possible that this competition could lead to the induction of antimicrobial activities within the community. Therefore, while *Serratia marcescens* 10A.1A.P216 individually did not have a high inhibition rate towards *R. solani,* we chose to also use this species as a third BCA to assess the possibility of this strain facilitating the production of antimicrobials in other two strains in the consortia. The serial dilution of C53 showed a combination of all 3 bacterial strains, confirming that they were able to grow together for 7 d without outcompeting each other (Supplementary Figure 2.6). Through understanding how these communities interact with the pathogen in the rhizosphere and their impact on plants, we can gain further insight into the mechanisms of these BCAs.

In planta experiments

Our results from the seedling damping off experiment indicate variability in the ability of BCAs to control disease in the soil. Pots inoculated with the pathogen had significantly lower germination and survival rates compared to those that were not inoculated (<0.001), however there were no significant differences between bacterial inoculation (Table 2.1). Shoot biomass was significantly impacted by *R. solani* AG2-2 inoculation (<0.001) and the interaction between bacterial community and *R. solani* AG2-2 (<0.05), however there was no significant difference attributed to bacterial inoculation. Treatment impacts on root biomass were significant for *R. solani* AG2-2 inoculation (<0.001) and the interaction between bacterial and *R. solani* AG2-2 (<0.001), with no significant impact of bacterial inoculation. Soil samples collected just before sowing seeds indicates that there was a significant (<0.05) difference in *R. solani* AG2-2 gene abundance in the growing media between inoculated and control pots, however there was not a significant difference in bacterial gene abundance (Supplementary Figure 2.7). The gene abundance of *R. solani* AG2-2 in soil samples collected 28 DPS showed significant differences due to pathogen inoculation (<0.001), however not for bacterial community inoculation (Figure 2.7). Total bacterial gene abundance in the soil also showed significant differences at 28 DPS due to *R. solani* AG2-2 inoculation (<0.05), with the inoculated pots having an overall lower Ct value per 10 ng of DNA, and therefore higher gene presence, compared to pots that were not inoculated with the pathogen. Firmicutes and gammaproteobacterial relative gene abundance were not significantly impacted by *R. solani* AG2-2 or bacterial community treatments. While we observed differences in bacterial abundance related to pathogen

inoculation, we cannot attribute this to successful colonization of the BCAs because our control pots experienced similar trends.

Table 2.1. Data collected for the (A) seedling damping off experiment 28 DPS and the (B) mature crown and root rot experiment 28 DPT.

A. Seedling Damping Off			B. Mature Crown & Root Rot		
Germination Rates (%)			Crown Rot (%)		
BCA	Diseased	Healthy	BCA	Diseased	Healthy
Control	68.75 ± 16.5	100 ± 0	Control	75 ± 46	0 ± 0
C1	43.75 ± 19.8	91.67 ± 12.60	C1	54 ± 47	0 ± 0
C15	66.67 ± 19.92	97.92 ± 5.89	C15	81 ± 35	0 ± 0
C53	64.58 ± 22.60	83.33 ± 23.57	C53	84 ± 35	0 ± 0
Total	60.94 ± 21.42	93.23 ± 14.58	Total	74 ± 41	0 ± 0
Survival Rates (%)			Root Rot (Scale 0-7)		
BCA	Diseased	Healthy	BCA	Diseased	Healthy
Control	27.08 ± 23.46	95.83 ± 7.72	Control	6.75 ± 0.71	0 ± 0
C1	28.13 ± 37.52	97.92 ± 5.89	C1	6.50 ± 0.93	0 ± 0
C15	29.58 ± 16.35	97.50 ± 7.07	C15	7 ± 0	0 ± 0
C53	39.58 ± 21.71	97.92 ± 5.89	C53	7 ± 0	0 ± 0
Total	31.09 ± 25.19	91.29 ± 6.42	Total	6.81 ± 0.59	0 ± 0
Shoot Biomass (g)			Shoot Biomass (g)		
BCA	Diseased	Healthy	BCA	Diseased	Healthy
Control	0.17 ± 0.15	0.81 ± 0.09	Control	1.14 ± 0.93	2.61 ± 0.21
C1	0.14 ± 0.12	0.49 ± 0.13	C1	0.97 ± 0.89	2.49 ± 0.23
C15	0.15 ± 0.13	0.65 ± 0.15	C15	0.74 ± 0.40	2.32 ± 0.24
C53	0.27 ± 0.24	0.53 ± 0.15	C53	1.02 ± 0.47	2.72 ±0.36
Total	0.18 ± 0.16	0.62 ± 0.17	Total	9.97 ± 0.67	2.53 ± 0.29
Root Biomass (g)			Root Biomass (g)		
BCA	Diseased	Healthy	BCA	Diseased	Healthy
Control	0.02 ± 0.02	0.14 ± 0.03	Control	0.29 ± 0.25	2.05 ± 0.34
C1	0.02 ± 0.02	0.10 ± 0.02	C1	0.45 ± 0.61	2.38 ± 0.84
C15	0.02 ± 0.02	0.08 ± 0.03	C15	0.11 ± 0.15	2.15 ± 0.64
C53	0.04 ± 0.04	0.07 ± 0.01	C53	0.18 ± 0.13	2.50 ± 0.45
Total	0.03 ± 0.02	0.10 ± 0.04	Total	0.26 ± 0.34	2.27 ± 0.58

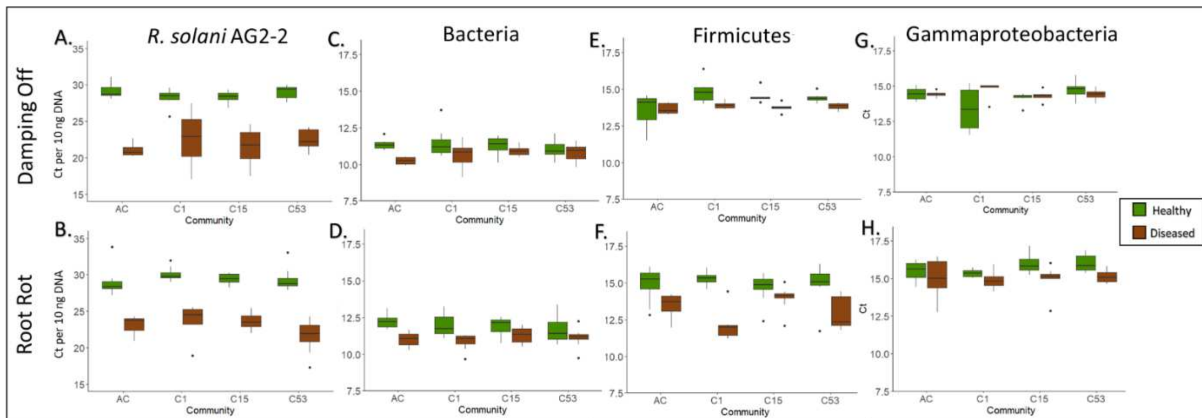

Figure 2.7. Quantitative PCR results for gene abundance of *Rhizoctonia solani* AG2-2 (A,B), bacterial (C, D), Firmicutes (G, H) and Gammaproteobacteria (G, H) in soil samples collected from the seedling damping off experiment (top) and the mature plant root rot experiment (bottom). Ct values are normalized based on 10 ng of DNA input. A lower Ct value indicates higher gene abundance.

For the mature plant experiment, the plants in growing media inoculated with pathogen showed high rates of crown rot, beginning after 10 DPT (Table 2.1). Crown rot was not observed on non-inoculated plants. There was high variation and no significant difference of percent crown rot due to bacterial community inoculation. Disease scoring of the roots at 28 DPT showed high levels of root rot for inoculated pots. There was no significant difference in root rot due to bacterial community inoculation. Dry biomass at 28 DPT of inoculated root and shoot samples was significantly (<0.001) lower than non-inoculated pots (Table 2.1). There was no significant difference in biomass due to bacterial inoculation of healthy or diseased plants. Soil samples collected from treatment pots immediately before 5-week-old sugar beet plants were transplanted indicates that there was a significant (<0.05) difference in *R. solani* AG2-2 gene abundance in the growing media between inoculated and control pots, however there was not a significant difference in bacterial gene abundance (Supplementary Figure 2.8). There was a significant difference in gene abundance for *R. solani* AG2-2,

bacterial and firmicutes (<0.001) and gammaproteobacterial (<0.01) due to the pathogen soil inoculation. However, we did not observe a significant impact of bacterial community inoculation for any of the genes (Figure 2.7).

The inoculation of C1, C15 and C53 did not impact the plant growth or visual disease severity for either the seedling or mature plant experiments. Similarly, we did not see an impact in the *R. solani* AG2-2 gene abundance related to bacterial inoculation. Considering the bacterial gene abundance, it's likely that the initial bacterial suspension was not able to colonize and establish in the growing media. We expected to see a high gene abundance of Firmicutes in C15 and C53, and a higher gene abundance of Gammaproteobacteria in C1 and C15, based on the bacterial strains included in the suspension. While we did not observe the trends that were expected in the qPCR results, further high throughput sequencing is required to confirm the ability of these bacterial strains to colonize the rhizosphere and potentially the roots. The plant rhizosphere contains specialized microbes that are adapted to specific niches. While the soil was autoclaved and the seeds were surface sterilized, it's probable that microbes remained in the soil, on the seeds, or as endophytes within the seeds. Seed endophytes can be well-adapted to the ecological niche and conditions within the plant, and therefore have a competitive advantage over introduced rhizosphere microbes (Hardoim et al., 2012). Therefore, when applying a BCA, there is a concern with successful colonization, as there will likely be competition with the existing plant-associated microbes. To further improve the efficiency of BCAs and to promote successful colonization of the plant rhizosphere, a seed coating method is recommended. Seed coating involves using a type of adhesive to enhance the performance of BCAs by

ensuring efficient delivery of the microbes into the rhizosphere and improve the likelihood of microbial survival (Ma, 2019; Rocha et al., 2019). Improving colonization of the rhizosphere and survivability could improve the competitive abilities of an inoculated BCA with the existing rhizosphere microbiota.

Our in-planta results for both the damping off and root rot experiments did not demonstrate effective pathogen control due to BCA inoculation. Our results are consistent with other studies in which compatibility and effectiveness in vitro did not translate to the soil conditions (Georgakopoulos et al., 2002; Köhl et al., 2019; Mazzola & Freilich, 2017). In a high-nutrient environment, such as laboratory growth media, bacterial populations face few growth inhibitors, and are therefore able to reproduce at high rates. In the rhizosphere environment, however, it's estimated to be 100 times less nutrient rich than laboratory media (Lugtenberg et al., 2017). It's possible that, when introduced to the rhizosphere, the bacteria were not able to reproduce within the rhizosphere to express the antifungal activity that we observed in vitro. The lower population in planta compared to in vitro assays could have multiple effects on the antagonistic abilities of the bacterial communities. Quorum sensing is bacterial signaling between and within species that results in regulation of gene expression, and it is dependent on bacterial population density (Babalola, 2010; Trivedi et al., 2021). Biofilm formation is mediated by quorum sensing and therefore population density. In BCAs, a biofilm can result in more efficient colonization of the rhizosphere due to a robust, stable population that is protected from environmental stress (Winkelströter et al., 2014), and can reduce pathogen infection through the establishment of a physical barrier (Walker et al., 2004). Antimicrobial metabolite production can also be dependent on population

density and biofilm formation (Köhl et al., 2019; Pierson III & Pierson, 2007). For example, an antimicrobial metabolite produced by *Pseudomonas fluorescens* was observed to require a certain threshold of cell density to control take-all wheat (Raaijmakers et al., 1999; Raaijmakers & Weller, 1998). Antimicrobial gene regulation based on population density could play a pivotal role in determining whether an antagonistic trait is induced or not.

The sugar beet variety, 4012RR, used in this study was highly susceptible to damping off and root rot caused by *Rhizoctonia solani* AG2-2. Years of sugar beet breeding have resulted in several varieties that are tolerant to this soil pathogen, potentially leading to tradeoffs in sugar yield or quality (Stevanato et al., 2019). The impact of breeding for resistant varieties has been observed to impact the plant-associated microbiome and consequently the gene expression in the rhizosphere communities (Mendes, Mendes, et al., 2018; Mendes, Raaijmakers, et al., 2018). The recruitment of specific rhizosphere microbiota in tolerant sugar beet contributes to the plant's ability to overcome pathogen infection (Kusstatscher et al., 2019). The use of a highly susceptible sugar beet variety in this study suggests that the BCAs were likely outcompeted by a rhizosphere community that would not normally confer any resistance to *Rhizoctonia solani*. It's possible that without microbiota that would normally reduce pathogenicity in the soil, the plants were unable to resist severe infection, even after a BCA was applied. To explore the impact of the crop-associated microbiota due to resistance breeding on the efficacy of these BCAs, further experimentation should be conducted using varieties of sugar beet with differing degrees of tolerance to the pathogen.

To further clarify the influence of these microbial inoculants on disease management and plant growth of sugar beet, additional experiments could be completed under different conditions. For example, the use of sterilized potting media compared to field soil has been observed to have a significant impact on the microbial community structure (Grunert, 2017) which can influence the efficacy of microbial inoculants (Salomon et al., 2022; Van Gerrewey et al., 2020). In healthy field soil, it's likely that the pathogen would not be able to establish as well as we observed in this study. The regulated conditions in the greenhouse also may not be a good representation of how these microorganisms would interact in the field because they are not required to adapt to changing conditions. The high variability between success in greenhouse and field studies indicates that greenhouse studies may not be efficient or reliable (Fukami et al., 2016). Therefore, field experiments using the same BCAs may result in positive results and should be further explored.

CONCLUSION

Sugar beet is an important crop that is susceptible to many soilborne diseases, which can have detrimental impacts on the crop's yield and quality. It is essential to identify durable, effective biological control agents (BCAs) to mitigate resistant pathogen populations and minimize impacts on non-target organisms. While combining multiple microbial strains into BCAs may offer diverse antagonistic mechanisms, challenges may arise in compatibility between species within the BCA and interactions with the existing rhizosphere microbiota. The in-planta evaluation of the BCAs in this study represents the complexity when translating laboratory results to real soil conditions. Furthermore, when evaluating BCA effectiveness in the field, interactions with the native microbiome

may adversely affect its ability to confer disease resistance. This research contributes to the discovery of a systems-based approach for future exploration into multiple strain BCA development for more effective control of soilborne pathogens.

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CHAPTER 3: UNDERSTANDING THE COMPLEX INTERACTIONS BETWEEN A BACTERIAL ANTAGONIST AND FUNGAL PATHOGEN OF SUGAR BEET

INTRODUCTION

Microbe-microbe interactions are being increasingly studied and exploited to promote plant health and improve disease management strategies for agricultural crops. Bacterial-fungal interactions in the soil can drive ecosystem functions and are especially complex in natural environments (Deveau et al., 2018). The direct relationships between these two groups can be extremely diverse. For example, bacteria can act as endofungal mutualists (Bonfante & Desirò, 2017) or in symbiosis with ectomycorrhiza (Deveau et al., 2007; Schrey et al., 2005). Other antagonistic species can exhibit bacterial mycophagy (Leveau & Preston, 2008) or produce antifungal compounds such as lipopeptides, organohalogenic metabolites, terpenes, or volatiles (Garbeva et al., 2014; Meena & Kanwar, 2015; Schmidt et al., 2017). Habitat modification can be an indirect means of antagonism, such as pH modification (P. K. Sharma & Gothalwal, 2017; Vylkova & Lorenz, 2014) or oxygen availability (Kreth et al., 2008). To add to the complexity, these direct relationships can vary across different environments and conditions (Ferreira et al., 2015; Lindsay & Hogan, 2014; Walker et al., 2004). A deeper understanding of the mechanisms that drive bacterial-fungal interactions could lead to the discovery of novel biological control organisms.

Mechanisms of antagonism against plant pathogens are extensively studied for their potential uses in agriculture (Berg, 2009; Frey-Klett et al., 2011). Biological control

species can promote plant health through priming of plant immune responses (Gupta & Bar, 2020; Nishad et al., 2020; Zaid et al., 2022) or target the pathogen directly through production of antibiotics (Helfrich et al., 2018), competition for resources (Zelezniak et al., 2015), or predation (Parratt & Laine, 2018). Biological control agents (BCAs) are exposed to a small percentage of pathogen population for a short period in the pathogen lifecycle (B. Duffy et al., 2003; Handelsman et al., 1990), therefore the use of antagonistic microbes can reduce the potential for pathogen resistance compared to synthetic fungicides.

Less studied is the response of the pathogen to BCA applications, and the potential for the pathogen to overcome the attack. Mechanisms of pathogen resistance to applied biologic could result from modification or export of antifungal toxins (Schoonbeek et al., 2002; VanEtten et al., 2001), interruption in antagonism signaling or population dynamics (B. K. Duffy & Défago, 1997; Schnider-Keel et al., 2000) habitat modification (Dutton & Evans, 1996), physically blocking competitors (Di Giorgio et al., 1996), or production of toxins (López-Díaz et al., 2018). Understanding the response of pathogens to counteract challenges from other organisms could be important in understanding the variability in success of biological control organisms (B. Duffy et al., 2003). The ability for a pathogen to quickly evolve to overcome competition could lead to a reduction in efficacy. The evolution of species during microbe-microbe interactions is an important consideration when evaluating the efficiency of a microorganism against a pathogen.

Rhizoctonia solani is an economically important soilborne fungal pathogen that can target a wide host range of agricultural crops. The species is separated into 14

anastomosis groups, which are vegetatively incompatible may require unique chemical fungicides to successfully control (Tsror, 2010). *R. solani* AG 2-2 IIIB is a devastating pathogen of sugar beet, *Beta vulgaris,* and has been reported to have economic yield losses of 25% in the U.S. (Kiewnick et al., 2001; Wolfgang et al., 2023). This saprophytic fungus produces sclerotia, which can survive in the soil or in debris until conditions are favorable, aiding in its ability to persist in agricultural fields through rotations with resistant crops (Misra et al., 2023). In sugar beet, *R. solani* AG2-2 IIIB causes damping-off, crown rot and root rot. There has been extensive research into the sugar beet microbiome, specifically because of the ability to suppress soil pathogens that can cause devastating yield losses to growers (Carrión et al., 2019). The sugar beet microbiome also plays a critical role in quality of the crop, as microbes involved in nitrogen cycling can influence root sucrose content (Wolfgang et al., 2023). *Rhizoctonia*-tolerant sugar beet breeding has been successful, however these varieties are commonly limited in other important traits, such as yield and quality (Wolfgang et al., 2023). Extensive studies have been conducted on using biological control organisms to control disease and promote crop quality in sugar beet (Ahmed et al., 2023; Farhaoui et al., 2022; Haque & Parvin, 2021; Nikolić et al., 2019; Shawki et al., 2020), however there is minimal research on the microbe-microbe interaction that’s taking place.

The goal for this study is to gain a deeper understanding on the fundamental biological processes underlying highly specialized antagonistic interactions between biocontrol bacteria and R. solani. This research should act as a foundation to further explore potential biological control agents and evaluate methods of pathogen response. We isolated and characterized 136 bacteria from the sugar beet agroecosystem in

Colorado, USA as well as three important pathogens of sugar beet: *Rhizoctonia solani* AG2-2 IIIB, *Fusarium oxysporum* and *Fusarium secorum.* Bacterial isolates were screened for pathogen inhibition using *in vitro* competition assays. We used dual culture assays, volatile assay, and microscopy to determine the antagonistic activity of B. XX against R. solani. We used RNAseq to dissect changes in the transcriptomic profiles of both the bacterial and fungal partners following the dual culture are two time-point. Our results indicate that a highly antagonistic strain of *Bacillus* uses a variety of mechanisms against *R. solani,* including the production of the antifungal lipopeptide surfactin; siderophores and biofilm production; and successfully removing essential nutrients from the environment. We also postulate that the defense mechanisms employed by *R. solani* under these conditions are focused on cellular metabolism and survival in a nutrient-depleted environment. This study can be a foundation for exploration into potential biological control agents produced by *Bacillus* species*,* as well as improve our understanding of fungal response mechanisms to bacterial challenge.

MATERIALS AND METHODS

Extraction and storage

Our methods for extraction and storage are described in detail in chapter 2. Briefly, soil samples from sugar beet agroecosystems were serially diluted and 136 bacterial isolates collected. Isolates were streaked twice to ensure purity, DNA was extracted using the DNEasy Ultra Clean Microbial Kit (QIAGEN, Hilden, Germany) and Sanger sequencing using primers 27F and 1492R was performed at Eurofins Genomics USA for strain level identification. Isolated strains were stored in glycerol in -80C freezer

until use. Fungal pathogens, RZC24 R-1, 102-6a and FOB220a, provided by Dr. Kim Webb, USDA-ARS (Sugar Beet Research Unit, Fort Collins, CO), were grown in potato dextrose media and stored in glycerol in -80C.

Competition assays

Based on previous screening of 136 bacterial isolates from the sugar beet agroecosystem, we selected a highly antagonistic strain against *R. solani*. This isolate was identified as *Bacillus velezensis* based on sanger sequencing. To identify the ability of the *B. velezensis* to inhibit growth of *R. solani*, we completed dual culture assays on ½ strength potato dextrose agar (PDA). A 7 mm agar plug of 1 week old *R. solani* culture was inoculated in the center of a petri dish and a 20 mm streak of *B. velezensis* P4.A7A inoculated 20 mm away from the center of the agar plug. The assays were completed in triplicate. Control plates were not inoculated with the bacteria. After 8 d, percent inhibition was calculated by comparing the growth of the treated plates to control plates (Figure 3.1).

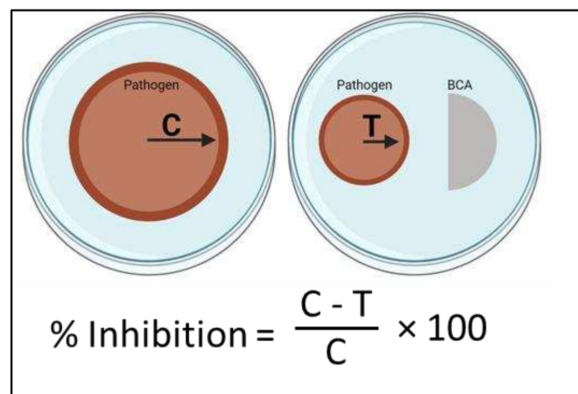

Figure 3.1. Percent inhibition calculation on dual culture assay plates.

Volatile assays

Volatile assays were completed using the sandwich plate method as described in Ebadzadsahrai et al (2020). 7 mm plugs of 1 week old *R. solani* culture was placed at the center of a ½ strength PDA plate. The plate cover was replaced with an agar plate streaked with *Bacillus velezensis.* The two plates were sealed with parafilm and incubated at 25C. Covers of control plates were also replaced with agar plate, without bacteria. After 8 days, mycelial growth of *R. solani* was compared with control plates and percent inhibition due to volatiles was calculated.

Microscopy

A thin layer of mycelium was collected from the fungal front approaching the inhibition zone and the opposite side (Figure 3.2). The mycelium was stained with either a neutral red dye or an Evan's blue dye, staining live and dead cells, respectively as described by Jiang et al.(2019). Briefly, 10 ul of dye was placed onto a slide with a thin layer of mycelium. The slides were incubated for 3-5 minutes, followed by gently washing the slides with distilled water 3-4 times. Microscopic images were taken on a Meiji EM-50L microscope (Meiji Techno San Jose, California) at 10x magnification.

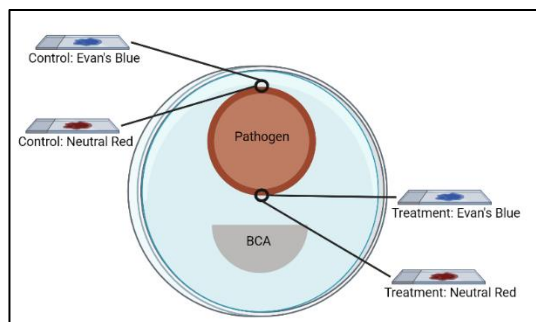

Figure 3.2. Mycelial samples used to make slides taken from the fungal front approaching the BCA and the opposite side. Mycelium was stained with a Neutral Red and an Evan's Blue dye to visualize viable and dead cells, respectively.

RNA extractions

RNA extraction and sequencing workflow is represented in Figure 3.3. A 7 mm plug of 1 week old culture from RZC24 R-1 was inoculated in the center of the plate on top of sterilized 0.22 mm filter paper. A single colony of bacterial isolate P4.A7A was streaked 20 mm away from the center of the agar plug, with no contact to the filter paper. Fungal control plates were set up in the absence of bacteria, and bacterial control plates were not inoculated with the fungus. Three replicates were completed for each bacterial-fungal interaction at each timepoint. Plates were incubated for 5 days at 25C. At day 3 and 5, a 15 mm diameter area of mycelium was collected from the fungal front approaching the inhibition zone and immediately flash frozen in liquid nitrogen. The peripheral mycelium zone was collected for fungal control plates. Frozen samples were ground using a pellet pestle in 1.5 ml microcentrifuge tubes until liquidized and immediately placed back into liquid nitrogen. Samples remained frozen until extractions. Bacterial colonies were also collected at day 3 and 5 and immediately flash frozen. Frozen samples were stored in -80C freezer. Fungal RNA was extracted using the QIAGEN RNEasy Plant Mini Kit (QIAGEN, Inc., Valencia, CA, USA). Bacterial RNA was

extracted using the Zymo DNA/RNA Miniprep Kit (Zymo Research, Irving, CA, USA). RNA concentration was quantified using Qubit HS RNA Assay Kit and the Qubit Flex Fluorometer (Thermo Fisher Scientific, Eugene, OR, USA). RNA integrity was evaluated using RNA ScreenTape analysis on an Agilent 4200 TapeStation (Agilent, Santa Clara, CA, USA). RNA samples were stored in -80C. Fungal RNA samples were shipped on dry ice to BGI (San Jose, CA, USA). Samples were treated for poly-A selection and sequenced on a DNBseq. Bacterial RNA samples were shipped on dry ice to Azenta Life Sciences (South Plainfield, NJ, USA) where a DNAse, library preparation and rRNA depletion was performed and RNA was sequenced on an Illumina Hiseq.

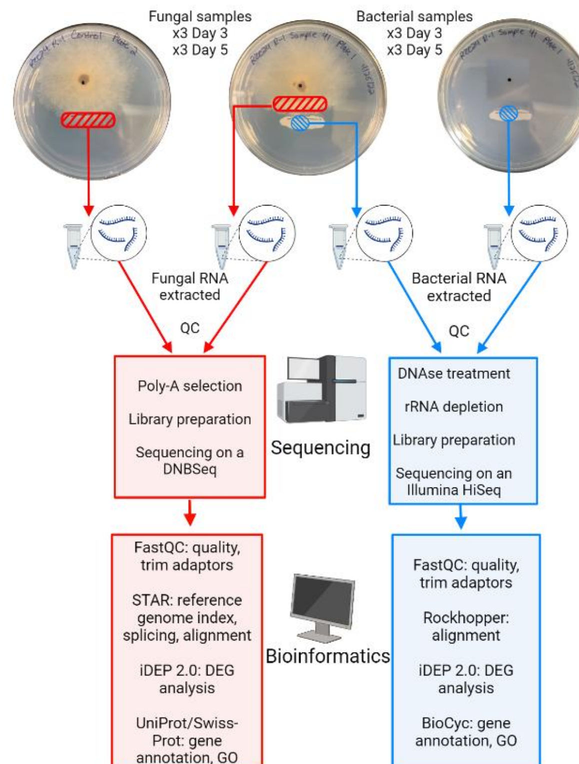

Figure 3.3. RNA extraction, sequencing and bioinformatics workflow for fungal and bacterial samples.

Bioinformatics

FastQC (Andrews, 2010) was used to identify quality of the forward and reverse reads for both bacterial and fungal samples. Illumina Universal Adapters were trimmed using cutadapt (Martin, 2011). Bacterial trimmed sequences were uploaded into Rockhopper (McClure et al., 2013; Tjaden, 2015, 2020) and aligned to *Bacillus amyloliquefaciens* FZB42 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA263650/) with 73%-87% alignment. Analysis of read counts and differentially expressed genes (DEGs) were analyzed using iDEP 2.0 with an FDR cutoff of 0.1 and minimum fold change of 2 (Ge et al., 2018). Gene annotation was completed using the BioCyc database (Karp et al., 2019) with the *Bacillus subtilis* subsp. *Subtilis* strain 168 complete genome (https://www.ncbi.nlm.nih.gov/biosample/SAMEA3138188/). Further functional group classification was completed with data from Kuntz et al. (1997).

Spliced Transcripts Alignment to a Reference (STAR) was used to index and splice the reference genome *Rhizoctonia solani* AG2-2 (https://www.ncbi.nlm.nih.gov/biosample/SAMEA7848368/) and align fungal sequences (Dobin et al., 2013),with about 85% alignment, to the reference genome. Analysis of read counts and differentially expressed genes (DEGs) were analyzed using iDEP 2.0 with an FDR cutoff of 0.1 and minimum fold change of 2 (Ge et al., 2018). Gene annotation and ontology was completed with UniProt/Swiss-Prot database (Bairoch et al., 2005) and filtered for fungal genes.

Statistical Data Analysis

All statistical analyses and visualizations were completed using R v 4.0.5 (R Core Team & Team, 2022). 'Deseq2' R package (Love et al., 2014) was used to identify differentially expressed genes for RNA seq data with a significance level of 0.05. 'Ggplot2' was used to visualize volcano plots (Wickham, 2016). Heat maps were made by calculating z-scores for each DEG and using the 'Pheatmap' package in R (Kolde, 2012).

RESULTS AND DISCUSSION

Competition Assays

The antagonistic effect of direct interaction or diffusible compounds produced by *B. velezensis* was evaluated in terms of reduced radial mycelial growth in dual culture assays. Bacterial suppression of fungal growth on day 3 was 54.95% ± 4.38%. The inhibition continued to increase with longer incubation time. On day 5, the percent inhibition was 66.11% ± 0.96%. Antagonism due to volatile compounds was measured based on sandwich plate assays where the pathogen and bacteria were sealed together, but on different agar plates. The inhibition of *R. solani* due to *B. velezensis* volatile compounds was low, with 6.27% inhibition after 8 days. Our results indicate that the antagonistic activity of *B. velezensis* is related to the production of diffusible metabolites and/or direct contact with the fungal mycelium.

B. velezensis has been widely studied as a model biological control organism against many agricultural diseases (Jiang et al., 2019; Meng et al., 2016; Nam et al., 2014). Extensive genome mining for this species has revealed numerous genes

involved in biocontrol and plant growth promotion (Alenezi et al., 2021). It has been observed to produce antibiotic metabolites, such as lipopeptides, peptides and polyketides (Rabbee et al., 2019; J. Wang et al., 2018), secrete cell wall degrading enzymes (Myo et al., 2019), form biofilms (Al-Ali et al., 2018), solubilize phosphate (Afzal et al., 2023) and produce indole-3-acetic acid (Hong et al., 2022a). *B. velezensis* strains can produce antifungal volatile compounds (VOCs) that inhibit the growth of certain pathogens (Choub et al., 2022; Z. Gao et al., 2017; Lim et al., 2017), however the effects of the VOCs can vary based on pathogen (T. Y. Kim et al., 2022; Li et al., 2020). Certain strains of *B. velezensis* can control a variety of fungal pathogens, including *Fusarium* sp., *Macrophomina phaseolina, Colletotrichum gloeosporioides* and *Phytophthora sojae* (Choub et al., 2022; Han et al., 2021; Hong et al., 2022b). One strain of *B. velezensis* was observed to be compatible with azoxystrobin and fluxapyroxad, two fungicides commonly used in *R. solani* control, further improving its potential as a biological control organism (G. Lee et al., 2023)

Microscopy

Microscopic images of *R. solani* hyphal samples showed partial cell death at the edge of the bacterial front, as indicated by partial neutral red stain (Figure 3.4). Microscopic analysis also revealed increased septation, branching of the hyphae and enlargement of vacuoles. Similar morphological changes in *R. solani* hyphae have been observed when challenged with *Serratia* sp., *Bacillus subtilis* and *Pseudomonas fluorescens* (Gkarmiri et al., 2015; Khedher et al., 2015; Thrane et al., 1999). The deformation in hyphae may result from antimicrobial metabolites targeting the cell wall or protection from changes in environment (Gkarmiri et al., 2015; N. Sharma & Sharma,

2008) Lipopeptides, common metabolites produced by *Bacillus* species, can disrupt cell membranes, leading to osmotic imbalance (T. Y. Kim et al., 2022). Given the dynamic nature of vacuoles, their altered size may be associated with the mitigation of osmotic stress (Veses et al., 2008).

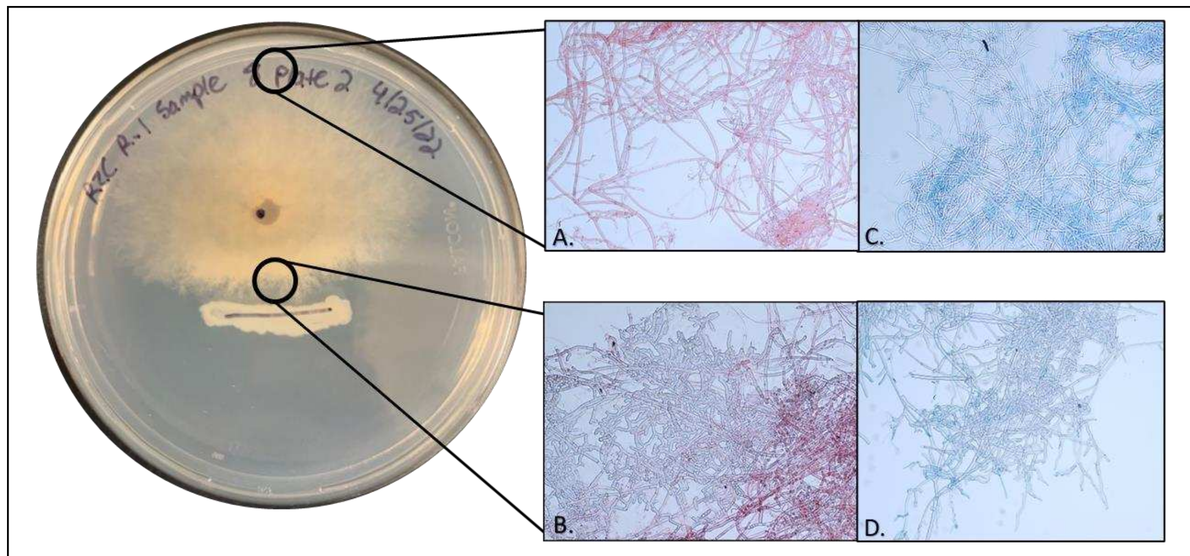

Figure 3.4. Microscopic images taken at 10x magnification from samples collected from mycelial growth at the back of the plate (A) and mycelial front facing the bacteria (B). Neutral red stain indicate viable cells. Increased branching, septation and cell death is visible in the sample closes to the bacteria.

Dual RNA-seq analysis of *Bacillus velezensis* P4.A7A and *Rhizoctonia solani* AG2-2 IIIB

*Bacillus v*elezensis P4.A7A gene expression

Total bacterial read counts per sample were between 5 million and 9 million reads (Supplementary Figure 3.1). For bacterial RNA-seq data, we observed significant over expression of 314 genes and under expression of 413 genes on day 3. DEGs increased after 5 days of interaction, with 818 genes being over expressed and 775 genes under expressed (Figure 3.5). Our results showed that both for the over and

under expressed genes there were a low number of genes that were shared between D3 (4 shared genes) and D5 (1 shared gene) (Supplementary Figure 3.2). Our results suggest the bacterial response to the pathogen is dynamic and becomes more stronger with time.

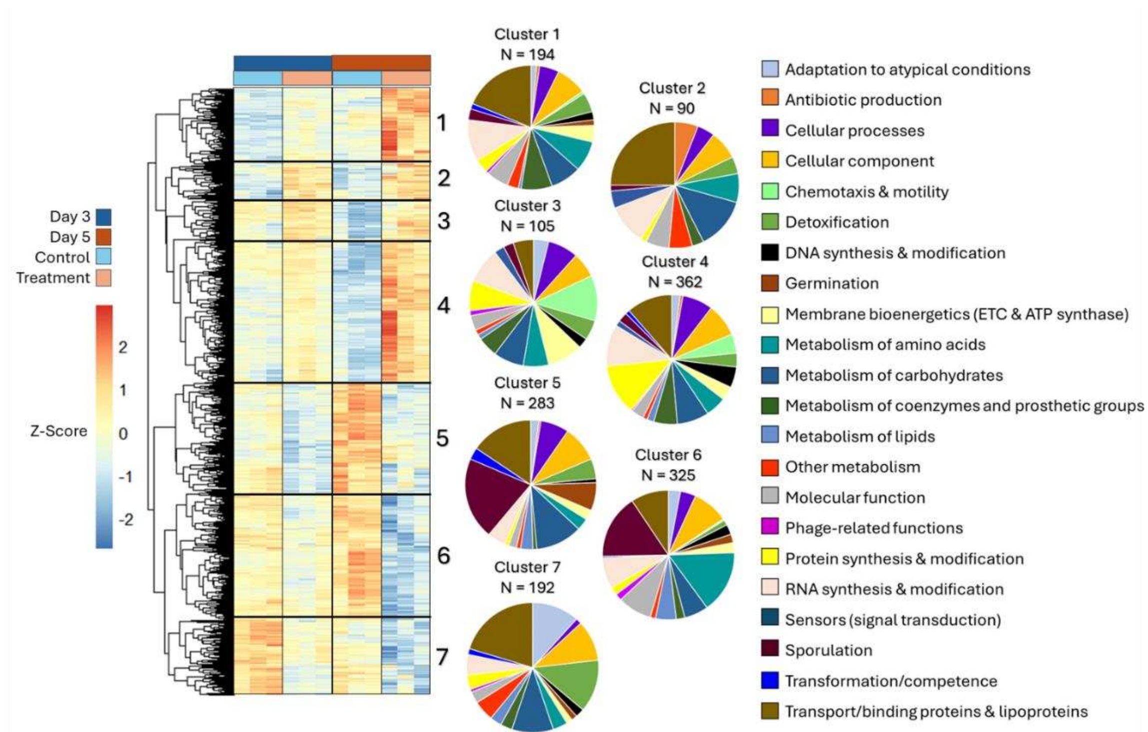

Figure 3.5. Heat map of z-scores based on Deseq2 analysis representing RNAseq data for *Bacillus velezensis* P4.A7A for day 3 and day 5. Pie graphs represent gene clusters taken from heat map data. There is an overexpression of antibiotic production (cluster 2) in D3 and D5 dual plate samples compared to controls. We also observed an over expression of protein synthesis related genes (cluster 1-4) in D3 and D5 samples on dual plates compared to control, which could indicate an increase in nutrient acquisition and metabolism. Our results also indicate an under expression of sporulation genes (cluster 5 & 6) in D3 and D5 dual plate samples, indicating that the bacteria are not under specific stress. There was also an under expression of detoxification genes, which suggests that the pathogen defense response does not involve the production of antibacterial toxins.

Genes that can explain the antagonistic activity of *Bacillus velezensis* P4.A7A

Siderophore production

Many bacterial species can produce siderophores, which are molecules that have a high affinity for iron, and therefore improve uptake of iron for that organism. Our results revealed an over expression in genes involved in siderophore production at both day 3 and day 5 (Table 3.1). Genes dhbA, dhbB, and dhbF involved in the production of siderophore, bacillibactin (Dunyashev et al., 2021), were overexpressed in both D3 and D5 compared to their respective control. At both D3 and D5, we observed the overexpression of genes involved in the utilization of hydroxamate siderophores including FhuBGC ABC transporter together with the FhuD (ferrichrome) or YxeB (ferrioxamine) encoding for substrate-binding proteins (Schneider & Hantke, 1993)Three gene (yclO, yclP, and yclQ) that are part of ABC transporter complex YclNOPQ were overexpressed both at D3 and D5. The product of these genes is involved in the selective binding of iron-free and ferric siderophore, petrobactin and petrobactin precursor 3,4-dihydroxybenzoic acid (3,4-DHB) its uptake inside the cells (Zawadzka et al., 2009). Among the over expressed genes for both D3 and D5 is feuA, feuB and feuC, together making up the iron ABC transporter complex FeuABC. This transporter is involved in import of the catecholate siderophores bacillibactin and enterobactin (Ollinger et al., 2006). Interestingly, increase intracellular iron through the transcription of the FeuABC transporter complex activates the KinB (also overexpressed at both D3 and D5) dependent synthesis of biofilm matrix components (Xu et al., 2019). We postulate this strategy is beneficial for competition with fungal pathogen and biofilm formation. As a biological control strategy, siderophore-producing organisms can

remove excess iron from the soil, depleting the bioavailable iron for pathogens (Rizzi et al., 2019). Iron is an essential micronutrient for cellular processes for many microorganisms, therefore the removal of iron from the environment can be growth-limiting or have detrimental effects (Da Silva & Williams, 2001). Biofilms can significantly improve the ability of siderophore producing bacteria to acquire iron (Rizzi et al., 2019), further interrupting essential processes in pathogenic microorganisms.

Table 3.1. *Bacillus* sp. P4.A7A genes involved in siderophore (orange) and antibiotic production (blue) after 3 and 5 days of co-culture with *Rhizoctonia solani.*

Symbol	Gene Name	D3 (log2)	D5 (log2)
bacA	prephenate decarboxylase	0.893366	1.753115
bacB	3-((4R)-4-hydroxycyclohexa-1, 5-dien-1-yl)-2-oxopropanoate isomerase	0.750776	0.912173
dhbA	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (2,3-dihydroxybenzoate biosynthesis)	1.130221	1.15863
dhbB	isochorismatase (2,3-dihydroxybenzoate biosynthesis)	1.014195	1.764517
dhbF	involved in 2,3-dihydroxybenzoate biosynthesis	0.612578	1.069355
feuA	iron-uptake system (binding protein)	0.992653	2.314296
feuB	iron-uptake system (integral membrane protein)	0.828568	2.433741
feuC	iron-uptake system (integral membrane protein)	0.9744	2.085145
fhuB	ferrichrome ABC transporter (permease)	0.862852	1.458457
fhuC	ferrichrome ABC transporter (ATP-binding protein)	0.904621	2.277512
fhuD	ferrichrome ABC transporter (ferrichrome-binding protein)	1.237765	3.264481
yclQ	ferrichrome ABC transporter (binding protein)	0.900848	3.205839
yxeB	ABC transporter (binding protein)	1.002939	2.175944
kinB	two-component sensor histidine kinase [Spo0F] involved in the initiation of sporulation	0.500874	0
yclO	ferrichrome ABC transporter (permease)	0.899805	0
yclP	ferrichrome ABC transporter (ATP-binding protein)	0.866286	0
albG	putative integral membrane protein involved in subtilosin production and immunity(albG)	0	-1.90006
dhbC	isochorismate synthase (2,3-dihydroxybenzoate biosynthesis)	0	1.419002
dhbE	2,3-dihydroxybenzoate-AMP ligase (enterobactin synthetase component E)	0	1.826286
bacC	bacilysin biosynthesis oxidoreductase	0	0.750776
sfp	surfactin production	0	1.153582
srfAA	surfactin synthetase / competence	0	0.61246
srfAC	surfactin synthetase / competence	0	0.838604

Antibiotic production

The production of antimicrobial metabolites is an effective mechanism of biological control for many *Bacillus* biological control species. Our results showed an over expression of genes involved in surfactin production at day 5 (Table 3.1). Three genes involved in surfactin production were over expressed at D5, which included two surfactin synthetase genes (srfAA and srfAC) and the sfp gene, which are all essential

for surfactin production (H.-S. Kim et al., 2000; Nakano et al., 1991). These genes encode 3 out of 5 genes that comprise the srfA operon, and therefore surfactin regulation (Y.-K. Lee et al., 2007). Surfactins are cyclic lipopeptides that can be essential for biofilm formation and directly cause cell death in microorganisms through the production of pores in the membrane (Falardeau et al., 2013). Surfactin is essential for motility and acts as signal for biofilm formation. Furthermore, purified surfactin A has shown strong antifungal activities against *Fusarium* and *Trichoderma* (Pérez-García et al., 2011; Sarwar et al., 2018). The artificial production and application of surfactin for disease control has been explored, however the high cost and low yield provides limitations for agricultural applications (Chen et al., 2015; Shaligram & Singhal, 2010).

Cellular processes

Cell division

Out of the 16 genes showing differential expression under cell envelope & cellular processes: cellular division category, 10 were overexpressed in day 5 compared to the control. Interestingly, a few of the overexpressed genes have a secondary role in virulence, biocontrol, or antibiotic susceptibility in other bacterial species. For example, glucose-inhibited division protein (gidA), is a translational regulator that plays an important role in the growth, cell division, capsule biosynthesis, and virulence of *Streptococcus* sp. (T. Gao et al., 2016; Rehl et al., 2013). In *Pseudomonas fluorescens*, gidA is reported to regulate the biosynthesis of antibiotic effective against *F. oxysporum* (W. Zhang et al., 2014). In *Lysobacter capsica*, gidA regulates biofilm production and motility that is essential for biocontrol activities (Zhao et al., 2022). The glucose-inhibited division gene (gidB) gene encodes for a methyltransferase enzyme that is responsible

for the susceptibility of Salmonella and Mycobacterium spp. against aminoglycoside antibiotics streptomycin and neomycin (Mikheil et al., 2012; Wong et al., 2011). In *B. anthracis*, the gene ftsX, which encodes the transmembrane domain of a putative ATP-binding cassette transporter, is a target of antibacterial chemokines (chemotactic cytokines) (Crawford et al., 2011).

Cell wall

Several genes involved in the biosynthesis of teichoic acid (including tagA, tagB, tagF, tagG, tagH, tagO) were overexpressed at D5 while genes involved in the biosynthesis of teichuronic acid [including tuaB, tuaC, tuaD, tuaE, tuaF, tuaG, tuaH (7 out of 8 genes in tua operon)] were under expressed both at D3 and D5. While both teichoic and teichuronic acid are essential cell wall components in *Bacillus* sp., the biosynthesis of teichuronic acid is accelerated during phosphate starvation (Bhavsar et al., 2004). Our results suggest that the bacteria grown with the fungal pathogen does not experience phosphate stress and therefore invests in the biosynthesis of a greater amount of teichoic compared to teichuronic acid. In accordance with this, we find under expression of two genes phoA (produces alkaline phosphatase A) (D5) and ylaK (produces phosphate starvation inducible protein) related to phosphate stress. Interestingly, in *Bacillus* sp. teichoic acid plays a major role in biofilm formation and maturation compared to alternate polymer, teichuronic acids (Bucher et al., 2015). We also observed the overexpression of genes involved in the Dlt pathway (dltB-D) that mediates the esterification of teichoic aid by D-alanine to modulate the structural properties of the membranes (Nikolopoulos et al., 2022).

Our results also revealed the overexpression of genes involved in the production of major host-encoded cell wall-lytic enzymes (autolysins; lytC and lytE) and modifier protein of major autolysin LytC (lytB) at D5. LytC are reported to distinguish the motile individuals from non-motile multicellular chains by influencing flagellar synthesis (Chen et al., 2009). In accordance with these results, we observed overexpression of genes related to flagellar synthesis and motility exclusively at D5. Autolysis mediated by lyt genes is also a novel and prophage-independent pathway for membrane vesicle production in *B. subtilis*, serving as a decoy for the cellular membrane to protect the living cells in the culture from membrane damage (Abe et al., 2021). Penicillin-binding proteins (PBPs) are a family of bacterial enzymes responsible for the synthesis of peptidoglycans via the polymerization of the glycan chain (transglycosylation) and the cross-linking of peptide chains (transpeptidation). β-Lactam antibiotics such as penicillin inhibit the transpeptidation reaction by covalently binding to the PBPs, resulting in cell lysis. Out of 16 genes involved in the production of PBPs in *Bacillus subtilis*, we observed differential expression in 12 PBP genes in D3 and/or D5. We didn't observe a clear pattern in the expression as 5 genes were over expressed and 6 under expressed at D5. Many of the PBPs play redundant roles in peptidoglycan synthesis and the diversity of PBPs allows maintenance of cell wall structure even in the presence of antibiotics (Zapun et al., 2009).

Sporulation

Peptide transport and sporulation signaling in *Bacillus* sp. is served by three ATP-binding cassette (ABC)-type peptide uptake systems termed dipeptide permease (Dpp), oligopeptide permease (Opp) and another peptide permease (App). Our results showed overexpression of genes involved in the OPP transport systems (genes oppA-F) at both

D3 and D5 compared to respective controls. We didn't notice any differential expression for most of the genes involved in Dpp and App transport systems. We also observed overexpression of rapA, rapD, and rapJ genes both at D3 and D5 compared to their respective controls. Rap phosphatases acts as response regulator and reduces phosphorylate thereby dephosphorylating Spo0F~P. We postulate that although the early steps of sporulation are partially initiated in the bacteria, the overexpression of rap genes controls the formation of Spo0F~P, that is required for the later stages of sporulation. Our study showed the under expression of genes (mostly at D5) from cge family (involved in the maturation of the outermost layer of the spore), cot family (involved in the production of inner and outer spore coat protein); spoIIIA locus (expressed in the mother cell during endospore formation and which are essential for the activation of σG in the forespore); spoII locus (required for the engulfment of smaller forespore by mother cells); spoIV locus (required for proper spore cortex formation and coat assembly); spoVA locus (required for the production of mature spores); sps locus (required for spore coat polysaccharide synthesis); and ssp locus (required for the production of small acid-soluble spore protein).

At D5, we observed the under expression of several genes involved in the information pathways that are reported to positively impact the sporulation process. These include sigF [RNA polymerase sporulation forespore-specific(early) sigma factor (σF) (SpoIIAC)]; sigG RNA polymerase sporulation forespore-specific (late) sigma factor (σG) (SpoIIIG)]; spoIIIC [RNA polymerase sporulation mother cell-specific (late) sigma factor (σK) (C-terminal half)]; gerE (transcriptional regulator required for expression of late spore coat genes); splA [transcriptional regulator of the spore photoproduct lyase operon (splAB)]; spoIIID (transcriptional regulator of σE - and σK -dependent genes); and

spoVT (transcriptional positive and negative regulator of σG-dependent genes). In contrast, we observed overexpression of two transcriptional regulators that are involved in the suppression of sporulation process including paiA [transcriptional repressor of sporulation, septation and degradative enzyme genes (aprE, nprE, phoA, sacB)]; sinR [transcriptional regulator of post-exponential phase responses genes (aprE, comK, kinB, sigD, spo0A, spoIIA, spoIIE, spoIIG).

Protein synthesis

There was a total of 46 differentially abundant genes related to the information pathways for protein synthesis at D5 (only 2 day 3) and most of those were over expressed (44 out of 46). These gene belonged to amioacyl-TRNA synthetases (leuS, proS, thrS, ytpR); initiation (infC); elongation (lepA, tunF, ylaG); structural constituent of ribosome (31 genes belong to rpl, rpm, and rps gene families). Ribosomal proteins are essential for translation and ribosome assembly (Fei et al., 2008; Zaher & Green, 2010) and therefore play an essential role in protein synthesis. Interestingly, rplK, encoding ribosomal protein L11 was over expressed in D5 and it has been associated with the general stress response of *B. subtilis* and essential for cell survival (Akanuma et al., 2012; S. Zhang et al., 2001). It has been postulated that rplA, rplV and rpmF, encoding ribosomal proteins L1, L22 and L32, respectively, contribute to cell reproduction and sporulation (Akanuma et al., 2012). Overexpression of various genes involved in protein synthesis is an indication of maximizing of growth rate that is an important fitness strategy for bacteria.

Chemotaxis and motility

Bacteria can sense the soluble compounds released by the fungi leading to the flagella mediated chemotaxis towards the fungal hyphae that excrete them (Deveau et al., 2018). Chemotaxis can be important for interactions with hosts and colonization, as well as essential for behaviors such as swarming, biofilm formation and ability to form colonies (Colin et al., 2021). At D5, we observed the overexpression of several genes involved in flagellar assembly and chemotaxis (Figure 3.6). These include genes involved in the filament formation [including flid (filament cap), hag (filament structure), flgK and flgK (junction protein)]; basal body formation [flgB, flgC, flhO (rod structure), fliF and fliE (basal ring)], and stator motor formation [motA and motB (involved in flagellar motor rotation)] (Mukherjee & Kearns, 2014; Zuberi et al., 1991). Our results showed the overexpression of methyltransferases (CheR) which catalyze the transfer of a methyl group from S-adenosylmethionine to the chemotactic receptors, the methyl-accepting chemotaxis proteins (mcpA, tlpA) (Kirsch et al., 1993; Stephens et al., 2006). Gene mcpA and ylxH that were overexpressed at D5 are reported to be involved in swarming motility (Kearns et al., 2004; Kearns & Losick, 2003). Two transcriptional regulators for flagellar assembly and chemotaxis, cheB (two-component response regulator-like [CheA] /methyl-accepting chemotaxis proteins-glutamate methylesterase) and yvyF (polypeptide putative transcriptional regulator of flagella formation) (Szurmant & Ordal, 2004), were overexpressed in D5. We also observed the overexpression of genes that are involved in protein secretion including fliQ, flhA, flhF, fliR, fliK, fliZ, and fliS genes. The product of these genes is reported to encode virulence factors in related bacteria species (Altegoer et al., 2018; Bouillaut et al., 2005; Carpenter et al., 1993;

Foynes et al., 1999; Iyoda et al., 2001). We postulate that motility is an essential component of this *Bacillus* sp. mechanism against *R. solani,* however, it is necessary for the bacteria to recognize the pathogen prior to the over expression of these genes. Along with the increase in siderophore production and protein synthesis, it's likely that the motility and ability to form a biofilm is enhancing the ability of the bacteria to acquire nutrients from the agar, and therefore deplete the environment for the fungal pathogen.

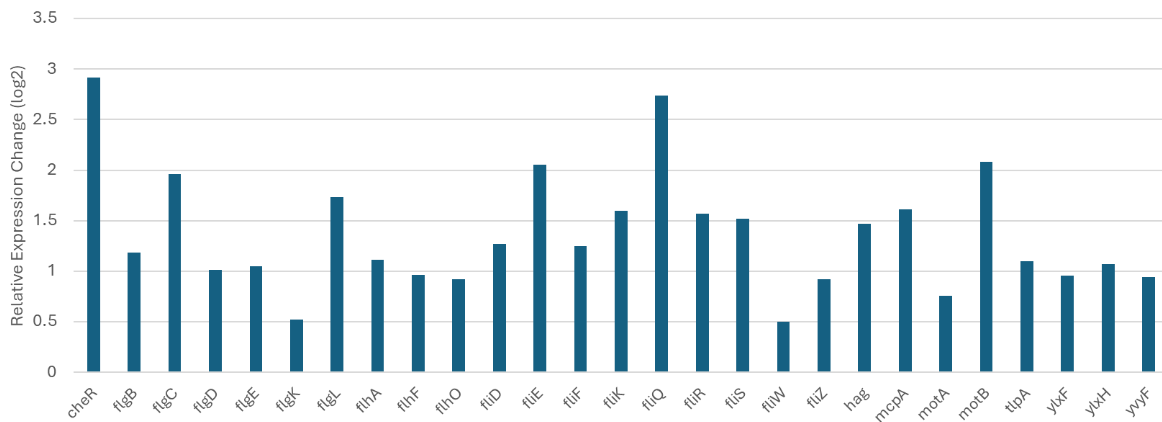

Figure 3.6. Relative change in expression of genes involved in motility and chemotaxis at day 5 compared to controls.

Adaptation to atypical stress

Our results revealed 63 genes related to the adaptation to atypical stresses that were differentially expressed in *Bacillus* sp. P4.A7A 26 in D3 and 37 in D5 compared to respective controls (Table 3.2). There was a total of 7, 8 and 14 under expressed genes at D3, D5, and both D3 and D5, respectively. There was a total of 1, 10, and 4 overexpressed genes at D3, D5, and both D3 and D5, respectively. Several genes related to general stress responses (including gsiB, ydaG, ytxG, ytxH, ytxJ, CTC, and gspA) were under expressed in D3 and/or D5, compared to controls. Most of the under

expressed genes belong to the σB regulon and are involved in functions to moderate stress management. They are likely involved in providing nongrowing cells with resistance to multiple stressors including heat, ethanol, and salt stress (Petersohn et al., 2001). Our results showed an overexpression of degR and its transcriptional regular degR both at D3 and D5 compared to the respective controls. The exoprotease production of Bacillus subtilis mediated by the degR gene is positively regulated by DegS-DegU two-component sensor histidine kinase involved in degradative enzyme and competence regulation (Ogura & Tanaka, 1997). Other overexpressed genes including yloA (overexpressed at both D3 and D5) and ywqC, ywqD, and ywqE (all overexpressed in D5) are reported to be involved in biofilm production (Pavez et al., 2023; Rodriguez Ayala et al., 2017). The ywqC-F (of which three out of four were overexpressed in D5) encodes for the tyrosine kinase PtkA (Gerwig et al., 2014) as well as to produce acidic exopolysaccharides (Mijakovic et al., 2003). Our results indicate that the presence of fungi is not perceived as a stress condition by *Bacillus* P4.A7A and therefore bacteria does not induce its molecular machinery to cope with starvation or environmental stress. On the other hand, *Bacillus* sp. P4.A7A selectively enhances mechanisms to antagonize the fungal pathogen via the production of degradative enzymes or biofilm production.

Table 3.2. Differentially expressed genes of *Bacillus* sp. P4.A7A involved in adaptation to atypical conditions after 3 and 5 days of co-culture with *Rhizoctonia solani.*

Gene Symbol	Gene Name	D3	D5
degR	degradative enzyme production	0.667262	1.447502
hfq	Hfq RNA chaperone	0.699023	1.188324
htpG	class III heat-shock protein (chaperonin)	0.706078	1.002736
yloA	fibronectin-binding protein	0.457606	1.114041
clpX	ATP-dependent clp protease ATP-binding subunit (class III heat-shock protein)	0	0.873759
cstA	carbon starvation-induced protein	0	0.929871
hit	Hit-like protein involved in cell cycle regulation	0	0.990178
rsbS	negative regulator of σB activity (antagonist of RsbT)	0	0.935566
rsbU	indirect positive regulator of σB activity (serine phosphatase [RsbV~P])	0	0.884014
sigL	RNA polymerase sigma factor (heat stress responsive)	0	1.322077
spxA	redox-sensitive regulator	0	1.399144
ywqC	capsular polysaccharide biosynthesis	0	1.932991
ywqD	capsular polysaccharide biosynthesis	0	0.820308
ywqE	capsular polysaccharide biosynthesis	0	0.957331
dps	stress- and starvation-induced gene controlled by σB	-2.38195	-2.26716
gbsA	glycine betaine aldehyde dehydrogenase (osmoprotection)	-1.28859	-1.98927
gbsB	alcohol dehydrogenase (osmoprotection)	-1.5634	-2.25761
gsiB	general stress protein	-2.43809	-2.33263
lonB	Lon-like ATP-dependent protease	-0.68053	-1.66334
ohrB	organic hydroperoxide resistance reductase B	-1.91712	-1.65192
rsbv	positive regulator of σB activity (anti-anti-sigma factor [RsbW])	-2.25339	-2.3623
rsbW	negative regulator of σB activity (switch protein/serine kinase [RsbV])	-2.09053	-1.6211
rsbX	indirect negative regulator of σB activity (serine phosphatase [RsbS~P])	-1.70374	-1.33676
ycdH	adhesion protein	-0.69779	-1.09257
ytxH	general stress protein	-1.56759	-1.32436
ytxJ	general stress protein	-1.35927	-1.92179
yvgO	exported stress induced factor(yvgO)	-2.31338	-2.07815
yvgO	exported stress induced factor(yvgO)	-2.31338	-2.07815
clpC	class III stress response-related ATPase (repressor of competence)	-0.85881	0
csbB	Stress response protein	-0.99243	0
ctc	general stress protein	-1.49962	0
gspA	general stress protein	-2.52088	0
rsbR	positive regulator of σB activity (interaction with RsbS)	-0.71015	0
ydaG	general stress protein	-1.80548	0
ytxG	general stress protein	-1.37535	0
clpQ	β-type subunit of the 20S proteasome	0	-1.04103
grpE	heat-shock protein (activation of DnaK)	0	-0.88218
pspA	phage shock protein A homolog regulator	0	-1.08576
yhaX	putative hydrolase(yhaX)	0	-0.77449
yhbH	factor involved in shape determination(yhbH)	0	-1.74605
ykoL	hypothetical protein(ykoL)	0	-2.7061
ypqP	capsular polysaccharide biosynthesis	0	-1.68801
yyxA	serine protease Do	0	-1.63693
yodU	capsular polysaccharide biosynthesis	0.658693	-1.32176

Detoxification

At D3, our results indicate that 8 genes involved in detoxification were overexpressed and 28 genes were under expressed (Figure 3.7). At D5, 24 genes were overexpressed, and 23 genes were under expressed, compared to controls. Notably, at D5, our results indicate an over expression of β-lactamase (penP), a defense mechanism that inactivates the antibiotic β-lactam before it can reach the penicillin-binding proteins, which is an indication that the bacteria are experiencing cell wall stress (Bucher et al., 2019). Interestingly, oxidative stress response genes, such as superoxide dismutase (sodA, sodF) and catalase (katE, katX) were under expressed in D3 and D5. Superoxide dismutase is involved in converting reactive oxygen species (ROS) into hydrogen peroxide (McCord & Fridovich, 1969; Padró et al., 2021), and catalase further catalyzes the breakdown of hydrogen peroxide into molecular oxygen and water (Broden et al., 2016). Alternatively, alkylhydroperoxide reductase genes (ahpC, ahpF) were both over expressed at D5. Alkylhydroperoxide reductase is also involved in the oxidative stress response of bacteria and is functionally similar to catalase (Cesinger et al., 2021). However, alkylhydroperoxide is specifically involved with scavenging the hydrogen peroxide that is naturally produced through cellular processes, while catalase can detoxify exogenous and high rates of hydrogen peroxide (Seaver & Imlay, 2001; Steele et al., 2010). Therefore, our results suggest that the *Bacillus* sp. P4.A7A is not under direct oxidative stress from the environment, however it is expressing mechanisms to detoxify endogenous hydrogen peroxide produced as a byproduct from cellular processes.

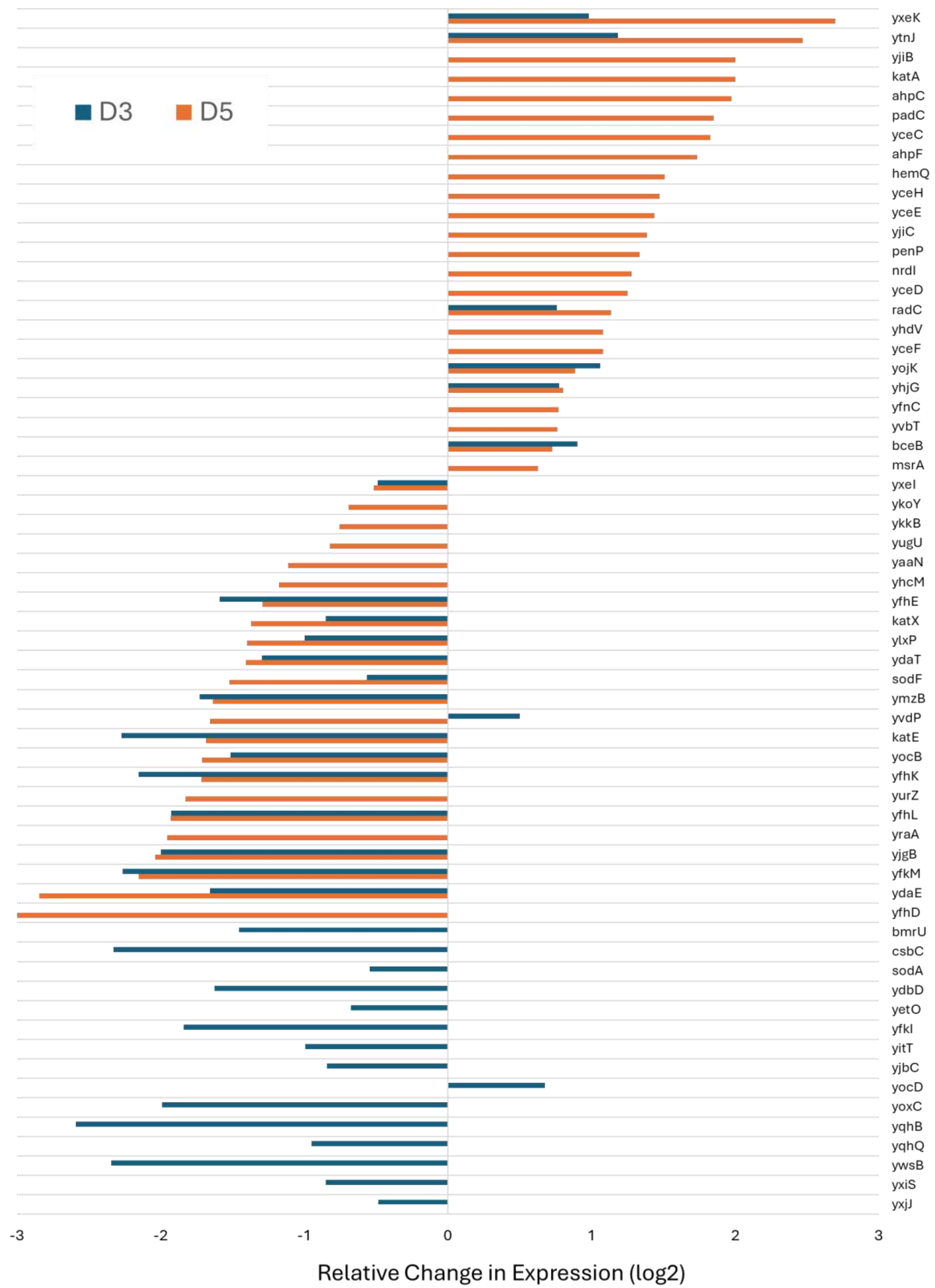

Figure 3.7. Relative change in expression of detoxification genes for day 3 (blue) and day 5 (orange).

Effects of *Bacillus* sp. P4.A7A on *Rhizoctonia solani* gene expression

Total fungal read counts were between 8,000 and 25,000 reads per sample (Supplementary Figure 3.3). Our results indicated that few genes were differentially expressed in the fungal genome due to the interaction with *Bacillus* sp. P4.A7A. Fungal RNA seq data on day 3 showed significant over expressed in 16 genes and under expressed in 18 genes. On day 5, we observed significant over expression in 90 genes and under expression in 124 genes (Figure 3.8). Due to the relatively few DEGs in the *R. solani* transcriptome, GO terms were used to classify the genes. The fungal genome showed few significant changes due to the interaction of *Bacillus* sp. P4.A7A. While DEGs in D3 did not have a significant grouping in terms of gene ontology, D5 genes were generally grouped into the cellulose catabolic process (GO:0030245) and the folic acid-containing compound biosynthetic process (GO: 0009396).

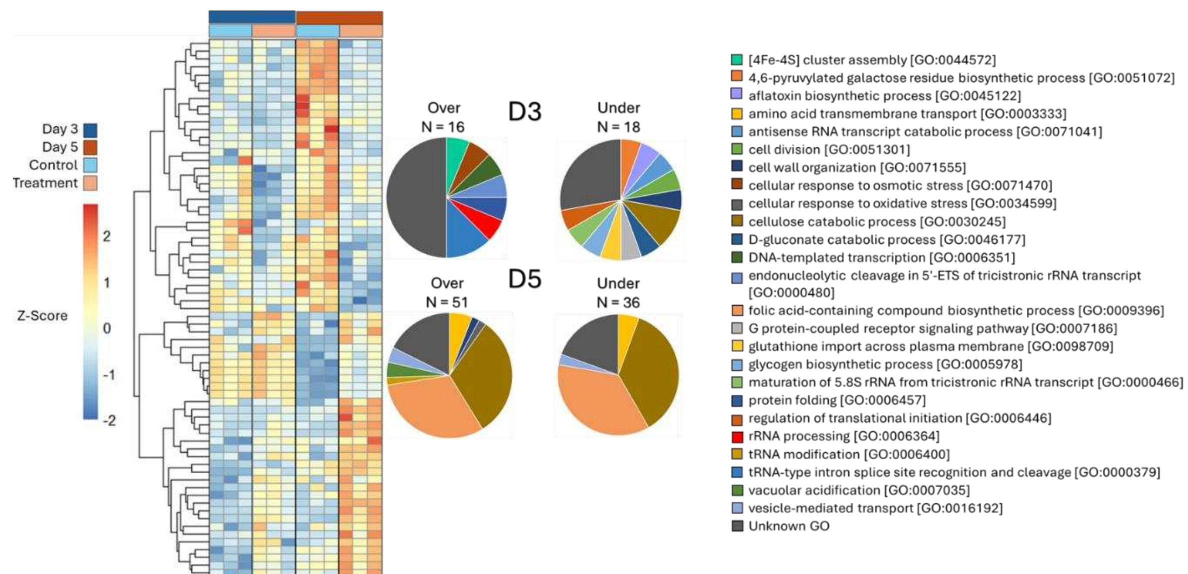

Figure 3.8. Heat map of z-scores based on Deseq2 analysis representing RNAseq data for *Rhizoctonia solani* AG 2-2 for 12 samples at D3 and D5 compared to controls. Pie graphs represent over and under expressed genes at D3 dual plate compared to control and D5 dual plate compared to control. At D3, genes involved in cellular processes and RNA synthesis were both over and under expressed. At D5, cellulose catabolism genes and metabolism genes were both over and under expressed, indicating a shift towards nutrient acquisition and survival.

At D3, we had an over expression of 5 genes involved in RNA synthesis, while at D5, we also observed an over expression of 1 gene. The genes that were over expression at D3 were specifically involved in rRNA processing (GO:0006364), tRNA-type intron splice site recognition and cleavage (GO: 0000379), endonucleolytic cleavage (GO: 0000480) and DNA-templated transcription (GO: 0006351), while tRNA modification (GO: 0006400) was over expressed at D5. The over expression in RNA synthesis after 3 days could be due to bacterial attack on the organism, prompting a defense strategy that involves enhancing RNA transcription.

The cellulose catabolic process is crucial for fungi to break down cellulose in plant cell walls and utilize it as a carbon source (B.-T. Wang et al., 2020). At D3, we observed the under expression of 2 genes involved in the cellulose catabolic process. In contrast, at D5, our results showed the overexpression of 16 genes and under expression of 10 genes related to the same process (Figure 3.9). The under expressed genes at D3 included xyloglucanase (cel74a) and the swollenin protein (swo1), known for breaking down cellulose and hemicellulose in plant cell walls (Benkő et al., 2008; Saloheimo et al., 2002). Notably, the proteolipid 3 protein (pmp3), associated with stress tolerance in fungi and yeasts, was overexpressed at D5 (Navarre & Goffeau, 2000). The increase in DEGs related to cellulose catabolism, while both under and over expressed, could be an indication of nutrient starvation and utilizing different pathways to acquire essential nutrients. It's important to acknowledge that the filter paper utilized on the agar plates might have influenced the cellulose catabolic processes differently compared to a culture grown directly on agar. Since filter paper primarily consists of cellulose, its presence could have affected the expression of DEGs as the pathogen utilized this pathway to obtain nutrients from the cellulose source. However, since filter paper was employed on both treated and control plates, any alterations in gene expression can still be attributed to the interaction with *Bacillus velezensis.*

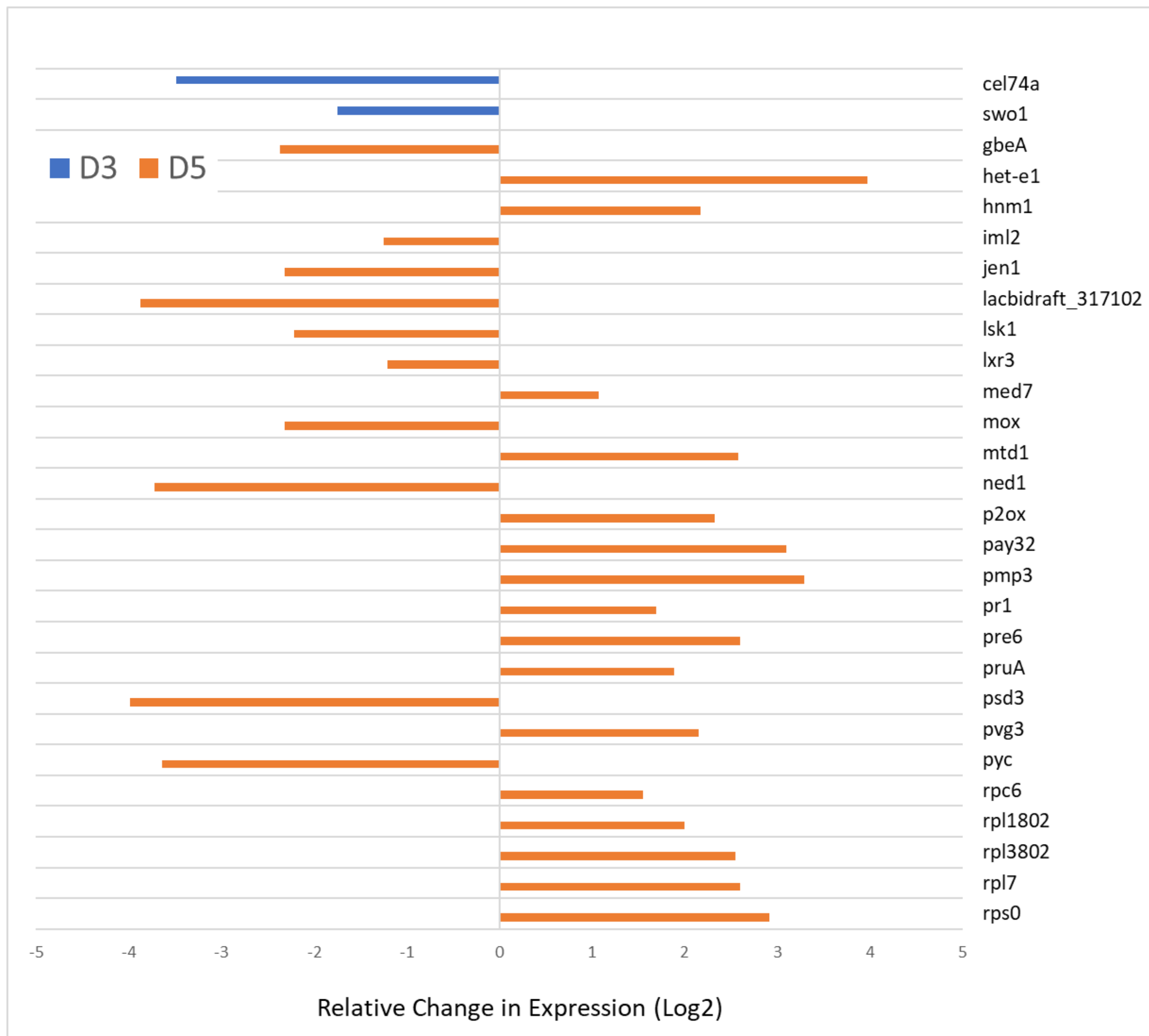

Figure 3.9. Relative change in expression of genes involved in the cellulose catabolic process (GO:0030245) at D3 (blue) and D5 (orange).

Genes involved in the folic acid-containing compound biosynthetic process were under expressed (12) and overexpressed (19) at D5, with no differentially expressed genes present in D3 (Figure 3.10). Folic acid biosynthesis generates folate, which plays a vital role in nucleotide and amino acid biosynthesis (Serrano-Amatriain et al., 2016). Folate has also been linked to the ergosterol pathway in fungi, which is critical for cell membrane structure (Dupont et al., 2012; Navarro-Martínez et al., 2006). Our findings do not reveal any discernible patterns in the folic acid biosynthetic process. However,

similar to cellulose catabolism, the significant changes in this metabolic pathway might indicate nutrient stress, prompting a metabolic shift essential for sustaining growth and ensuring survival.

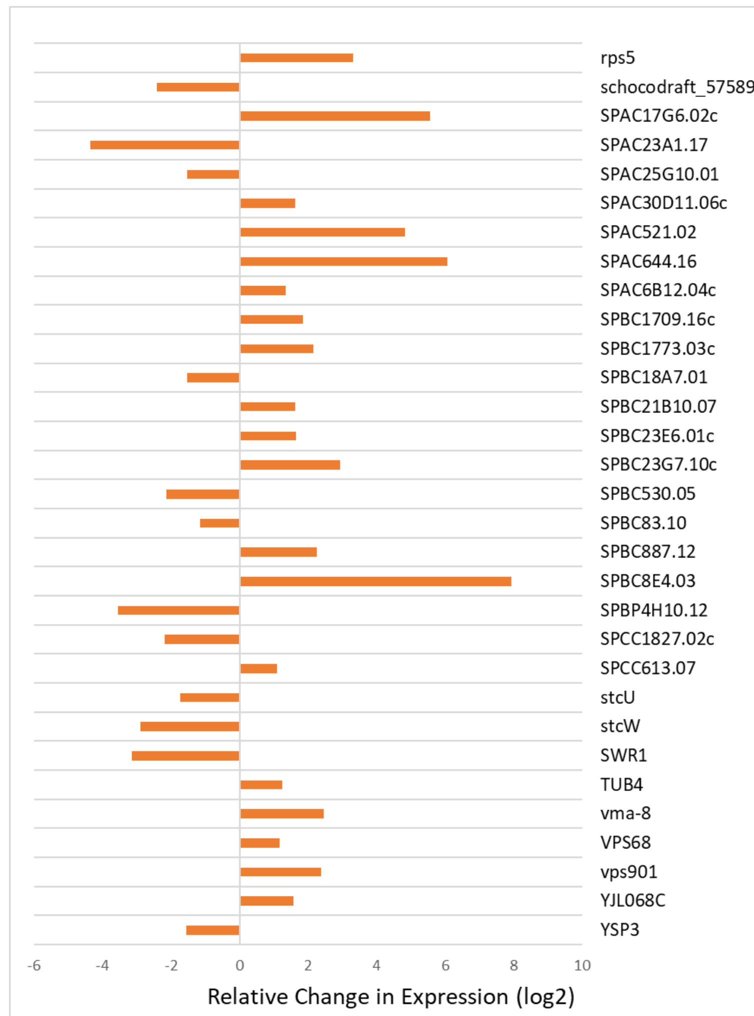

Figure 3.10. Relative change in expression of genes involved in the folic acid-containing compound biosynthetic process (GO: 0009396) at D5.

A few genes (fmpE, Pvg3, and cpr6) involved in stress mitigation were over expressed at both D3 and D5. Gene fmpE encodes fumipyrrole biosynthetic protein responsible for increasing cellular growth and decrease in sporulation and is associated with the cellular response to osmotic stress (Macheleidt et al., 2015). Our microscopic

analysis revealed an increase in the vacuole size within hyphae challenged with *B. velezensis,* suggesting a potential response to osmotic stress. Gene Pvg3 is a meiotically unregulated gene that encodes Golgi galactosyl xylosyl protein 3-beta-galactosyltransferase and is reported to provide resistance against a range of abiotic stresses (Andreishcheva et al., 2004; Martín-Castellanos et al., 2005). Gene cpr6 encodes Rotamase D protein whose abundance increases in response to DNA replication stress (Tkach et al., 2012). The overexpression of genes fmpE, Pvg3, and cpr6 at both D3 and D5 suggests the pathogen was under significant stress due to the interaction with BCA.

Vacuoles and vesicles are known to sequester secondary metabolites to protect host cells from self-toxicity. In fungi, these cellular structures also contain enzymes involved in secondary metabolism such as the biosynthesis of nonribosomal peptides, β-lactam antibiotic penicillin and polyketide aflatoxin (Chanda et al., 2009). At D5 there was an overexpression of genes associated with the maintenance of vacuolar acidification [GO:0007035] [vma-8 (encoding V-type proton ATPase subunit D) and vps68 (encoding vacuolar protein sorting associated protein)] and vesicle-mediated transport [GO:0016192] [vps901 (encoding vacuolar protein sorting associated protein) and YJLO68C (encoding S-formylglutathione hydrolase)]. In addition, two copies vacuole membrane gene, SPAC30D11.06c that encode the organic solute transmembrane transporter was overexpressed at D5. This protein is reported to provide resistance against stress conditions (D.-U. Kim et al., 2010; Liu et al., 2018).

Our findings suggest that following a 5-day co-culture with the BCA, the pathogen faced challenges in its survival and growth. Notably, there was a significant

increase in the number of DEGs associated with metabolism compared to D3. The fungal response corresponds with the mechanisms of the BCA, such as competing for nutrients, maintaining metabolic pathways and mitigating antifungal metabolites. Our results are consistent with other studies of fungal pathogens after exposure to a BCA. In a study examining the impact of *Serratia* sp. attack on *R. solani,* transcriptomic data 72 hours post infection revealed an overexpression in metabolism-related genes and the activation of defense strategies to mitigate oxidative stress (Gkarmiri et al., 2015). Similarly, *Fusarium* shared similar results, where genes involved in metabolic and cellular processes were both over and under expressed after co-culture with *Streptomyces* sp. (Strub et al., 2021). Overall, the fungal response to BCA interaction shifted towards metabolic pathways for growth and survival; however, this response appeared relatively weak and variable compared to the BCA's strong antagonistic capabilities.

CONCLUSION

The identification and application of biological control agents (BCAs) against fungal pathogens is a key strategy in mitigating fungicide resistance and enhancing crop health. Transcriptomic analysis offers valuable insights into both the mechanisms of BCAs and the responses of pathogenic fungi. In our investigation, *Bacillus velezensis* demonstrates a diverse array of strategies against *Rhizoctonia solani* in vitro, including siderophore production, biofilm formation, surfactin production, and an increase in protein synthesis, indicating the bacteria's ability to scavenge nutrients from the environment. Conversely, when confronted with this multifaceted attack from the bacteria, *R. solani* adapts by shifting towards pathways involved in stress mitigation and

nutrient acquisition. A thorough understanding of the interaction between bacteria and fungi in pathogen control holds significant promise for enhancing disease management in agriculture.

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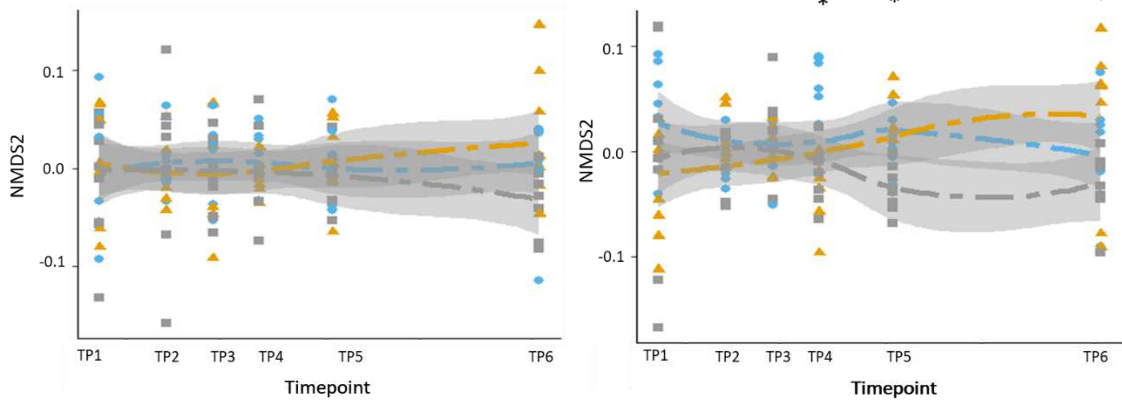
APPENDIX

	Lingle, WY			North Platte, NE		
Drivers	Sum of Squares	F-value	P-value	Sum of Squares	F-value	P-value
Timepoint	0.49469	11.3734	4.55E-09	3.7549	90.31	2.20E-16
Treatment	0.07321	4.208	0.01702	0.2679	16.105	6.13E-07
Timepoint:Treatment	0.11298	1.2987	0.23818	1.2388	14.896	2.20E-16

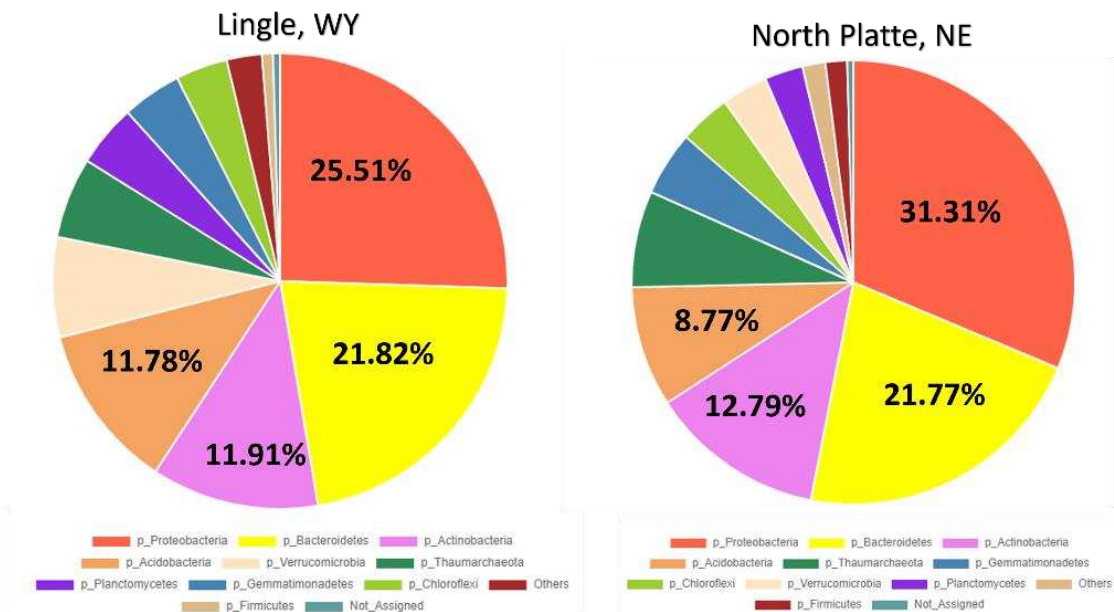
Supplementary Figure 1.1 The effects of timepoint, treatment and their interaction on the alpha diversity of samples within the two experimental sites based on ANOVA.

Lingle, WY			North Platte, NE		
Timepoint 1			Timepoint 1		
R^2	F-value	P-value	R^2	F-value	P-value
0.0946	1.1	0.215	0.105	1.23	0.143
Timepoint 2			Timepoint 2		
R^2	F-value	P-value	R^2	F-value	P-value
0.0704	0.796	0.951	0.0708	0.801	0.862
Timepoint 3			Timepoint 3		
R^2	F-value	P-value	R^2	F-value	P-value
0.0708	0.8	0.971	0.094	1.04	0.25
Timepoint 4			Timepoint 4		
R^2	F-value	P-value	R^2	F-value	P-value
0.0891	1.03	0.308	0.188	2.21	0.001*
Timepoint 5			Timepoint 5		
R^2	F-value	P-value	R^2	F-value	P-value
0.0803	0.917	0.922	0.198	2.59	0.001*
Timepoint 6			Timepoint 6		
R^2	F-value	P-value	R^2	F-value	P-value
0.0862	0.991	0.492	0.269	3.85	0.001*

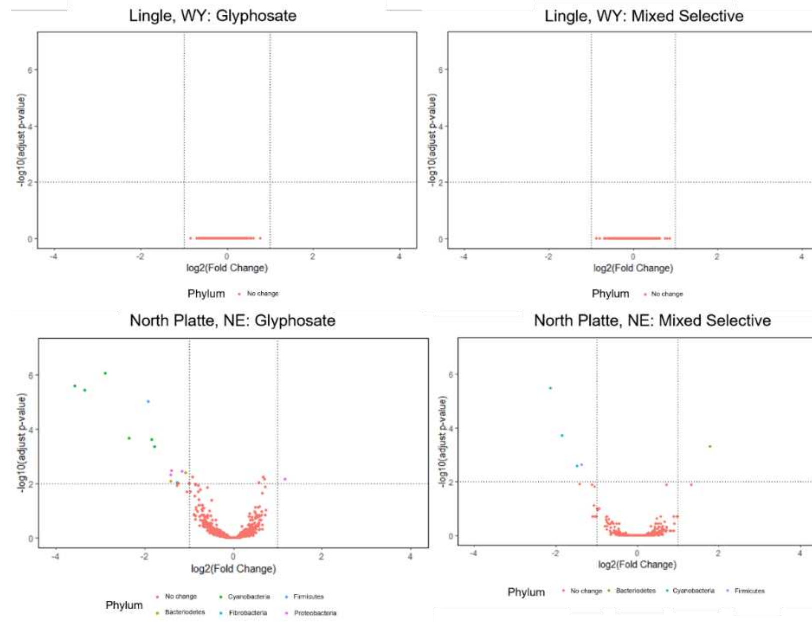
Supplementary Figure 1.2 PERMANOVA results showing the impact of treatment on the soil bacterial communities at each timepoint.



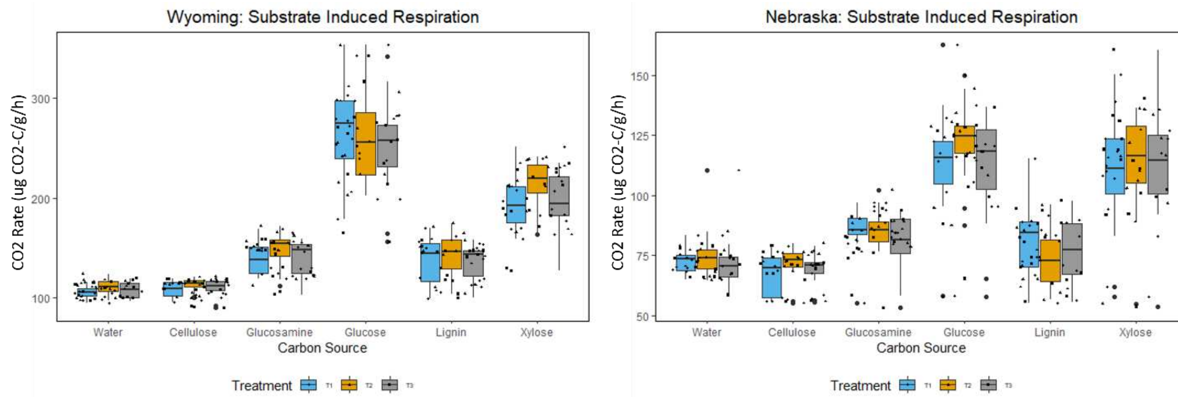
Supplementary Figure 1.3 Glyphosate (blue), mixed selective herbicide (orange) and control (gray) treatments are represented as scatter plots with standard error shading. Beta diversity is represented by Axis 2 of NMDS ordination plots constructed for each timepoint for the Lingle, WY site (B) and the North Platte, NE site (D). Significant differences between sample communities based on PERMANOVA are represented by an Asterix (*). A significance level of α = 0.05 was used for statistical analysis.



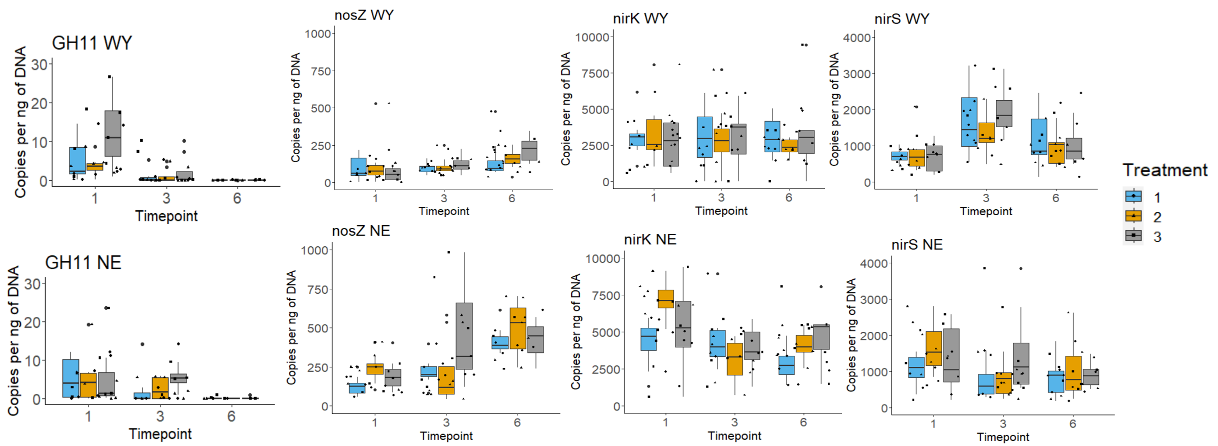
Supplementary Figure 1.4 Pie chart representing the percent relative abundance of each phylum for the combined treatments plots at the first sampling point at Lingle, WY (left) and North Platte, NE (right) sites.



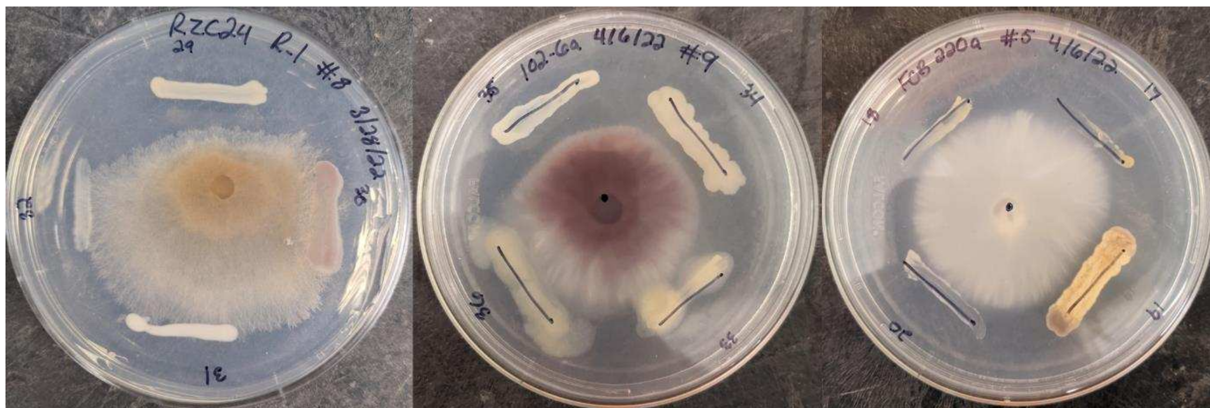
Supplementary Figure 1.5 Volcano plots representing the total enriched (right side) and depleted (left side) OTUs for the Wyoming (top) and Nebraska (bottom) sites.



Supplementary Figure 1.6 Carbon substrate induced microbial respiration for the Lingle, Wyoming (left) and North Platte, Nebraska (right) plots at the third sampling timepoint.



Supplementary Figure 1.7 Qualitative PCR data demonstrating functional gene presence in soil samples for nosZ, nirK, nirS and GH18 at timepoints 1, 3 and 6 for Lingle, Wyoming (top) and North Platte, Nebraska (bottom).



Supplementary Figure 2.1 Preliminary competition assays using 4 different bacterial isolates per nutrient agar plate.

Target	Primer Pair	Forward	Reverse	Annealing Temperature
AG2-2	AG2_2F/AG2_2R	GGCTCYRTTARTTTGGAG	TGTGAAGCTGCAAGAACC	55C
Total bacteria	Eub338/Eub518	ACTCCTACGGGAGGCAGCAG	ATTACCGCGGCTGCTGG	53C
γ-Proteobacteria	Gamma395F/Gamma871R	CMATGCCGCGTGTGTGAA	ACTCCCCAGGCGGTCDACTTA	54C
Firmicutes	Lgc353/Eub518	GCAGTAGGGAATCTTCCG	ATTACCGCGGCTGCTGG	60C

Supplementary Figure 2.2 Table of primers used for quantitative PCR for the in-planta experiments.

West

DO_4012_C53_RZC_7	DO_4012_AC_RZC_1	DO_4012_AC_RZC_4	DO_4012_C1_NR_6	DO_4012_C1_RZC_8	DO_4012_C15_NR_8	DO_4012_C15_NR_2	DO_4012_C1_RZC_2
DO_4012_C15_RZC_1	DO_4012_AC_RZC_8	DO_4012_C1_RZC_7	DO_4012_AC_NR_7	DO_4012_C15_RZC_7	DO_4012_C1_NR_5	DO_4012_C53_RZC_2	DO_4012_AC_NR_1
DO_4012_AC_NR_3	DO_4012_C1_NR_2	DO_4012_AC_NR_2	DO_4012_C15_NR_1	DO_4012_C53_RZC_8	DO_4012_AC_NR_8	DO_4012_AC_RZC_6	DO_4012_C15_NR_4
DO_4012_C53_NR_2	DO_4012_C53_RZC_4	DO_4012_AC_RZC_2	DO_4012_AC_RZC_7	DO_4012_C1_NR_3	DO_4012_C15_NR_7	DO_4012_C15_RZC_8	DO_4012_C15_NR_6
DO_4012_C15_NR_5	DO_4012_C1_RZC_3	DO_4012_C1_RZC_6	DO_4012_C1_NR_8	DO_4012_C53_NR_7	DO_4012_C53_NR_3	DO_4012_C53_NR_5	DO_4012_AC_RZC_3
DO_4012_C15_RZC_3	DO_4012_AC_NR_4	DO_4012_C1_RZC_5	DO_4012_C53_NR_1	DO_4012_C15_RZC_2	DO_4012_C53_RZC_1	DO_4012_C1_NR_1	DO_4012_C53_RZC_6
DO_4012_C15_RZC_5	DO_4012_C53_RZC_3	DO_4012_AC_RZC_5	DO_4012_C1_RZC_4	DO_4012_C15_RZC_6	DO_4012_C53_NR_8	DO_4012_C53_NR_4	DO_4012_C1_RZC_1
DO_4012_C53_RZC_5	DO_4012_C53_NR_6	DO_4012_C1_NR_7	DO_4012_AC_NR_5	DO_4012_AC_NR_6	DO_4012_C15_RZC_4	DO_4012_C15_NR_3	DO_4012_C1_NR_4

East

DO: Damping off
4012: Sugar beet variety
AC/C1/C15/C53: Bacterial BCA inoculated or absent
RZC/NR: *Rhizoctonia solani* inoculation or absent

Supplementary Figure 2.3 Treatments and complete randomized design in the greenhouse for the seedling damping off experiment.

West

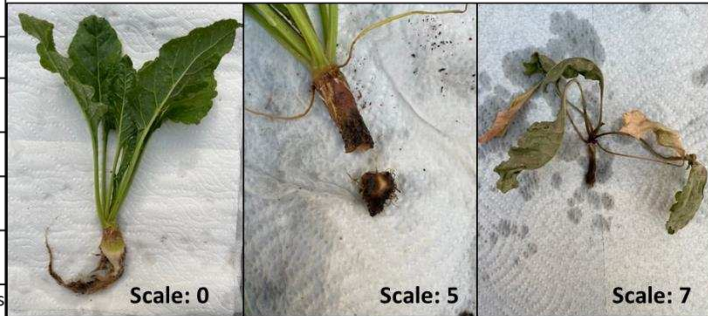
MAT_4012_AC_RZC_7	MAT_4012_C53_RZC_7	MAT_4012_C15_NR_4	MAT_4012_AC_RZC_5	MAT_4012_C53_NR_6	MAT_4012_C15_NR_3	MAT_4012_AC_NR_8	MAT_4012_C53_RZC_6	MAT_4012_AC_NR_7	MAT_4012_C15_NR_1	MAT_4012_AC_RZC_4
MAT_4012_C1_RZC_5	MAT_4012_C53_RZC_1	MAT_4012_AC_RZC_2	MAT_4012_C1_NR_3	MAT_4012_C53_NR_8	MAT_4012_C53_NR_2	MAT_4012_C15_RZC_7	MAT_4012_C15_RZC_4	MAT_4012_C1_RZC_7	MAT_4012_C15_RZC_5	MAT_4012_C1_RZC_3
MAT_4012_C1_RZC_1	MAT_4012_C1_NR_4	MAT_4012_C53_RZC_4	MAT_4012_C1_NR_2	MAT_4012_C53_RZC_5	MAT_4012_C1_RZC_6	MAT_4012_AC_NR_1	MAT_4012_C1_NR_1	MAT_4012_C15_NR_2	MAT_4012_C1_NR_5	MAT_4012_C53_RZC_2
MAT_4012_C53_RZC_3	MAT_4012_C53_RZC_8	MAT_4012_C1_NR_6	MAT_4012_C53_NR_4	MAT_4012_C15_RZC_8	MAT_4012_AC_RZC_1	MAT_4012_C15_NR_6	MAT_4012_C1_RZC_2	MAT_4012_C1_NR_8	MAT_4012_C1_NR_7	MAT_4012_C53_NR_1
	MAT_4012_C15_NR_7	MAT_4012_AC_NR_4	MAT_4012_AC_NR_5	MAT_4012_C1_RZC_8	MAT_4012_C15_RZC_6	MAT_4012_C15_RZC_2	MAT_4012_AC_NR_6	MAT_4012_C15_RZC_1	MAT_4012_C53_NR_3	MAT_4012_AC_NR_2
	MAT_4012_AC_NR_3	MAT_4012_C15_NR_8	MAT_4012_C53_NR_5	MAT_4012_AC_RZC_3	MAT_4012_AC_RZC_8	MAT_4012_C53_NR_7	MAT_4012_C15_RZC_3	MAT_4012_C1_RZC_4	MAT_4012_AC_RZC_6	MAT_4012_C15_NR_5

East

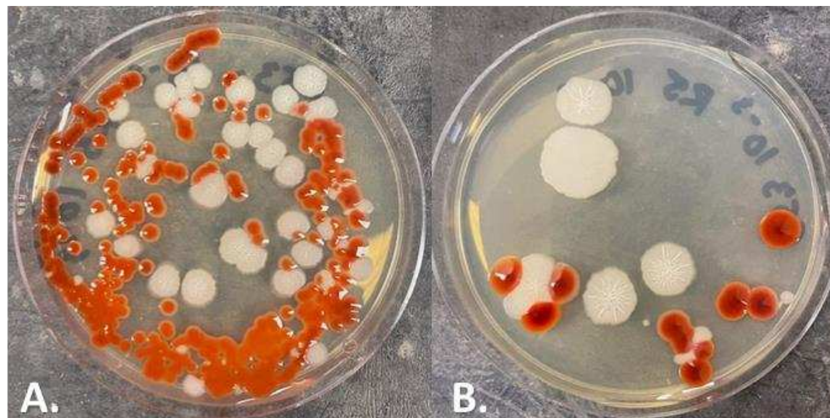
MAT: Mature
4012: Sugar beet variety
AC/C1/C15/C53: Bacterial BCA inoculated or absent
RZC/NR: *Rhizoctonia solani* inoculation or absent

Supplementary Figure 2.4 Complete randomized design in the greenhouse for the mature root and crown rot experiment.

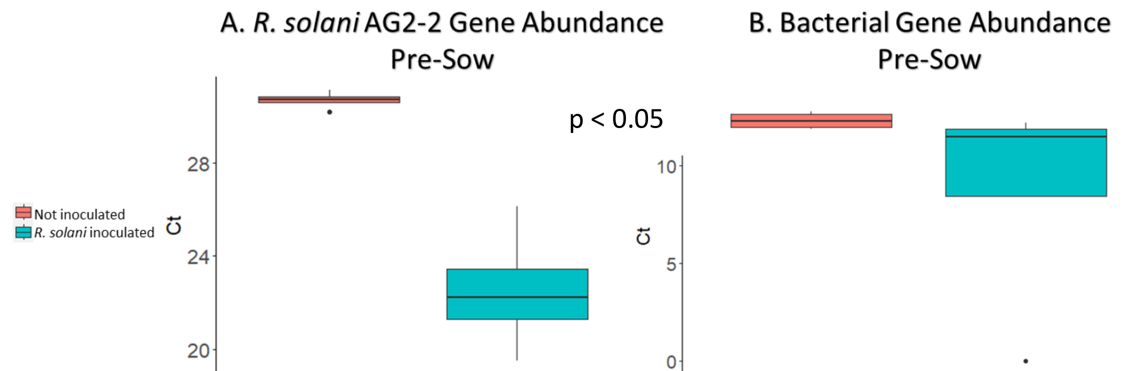
Scale	Description
0	No lesions
1	Very small dry lesions
2	Wet small circular active lesions (<5% of beet surface area)
3	Black epidermis and white interior of root
4	Black epidermis, yellow region adjacent and white center with distinct edges
5	Black penetrating into interior of root and center is yellow or white with indistinct edges
6	Lesions cover 75-100% of root surface area, center is brown and soft, few green leaves persist
7	Completely dead crown and shriveled root



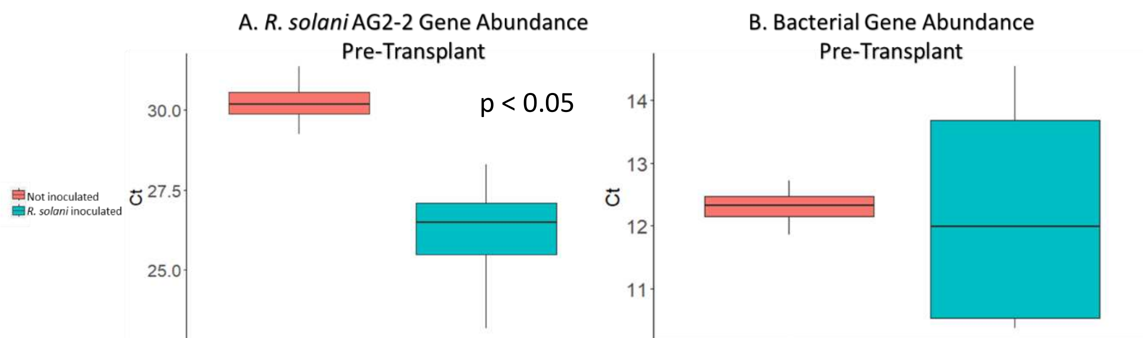
Supplementary Figure 2.5 Root disease scale ratings from the USDA-ARS Sugar Beet Research Unit (Fort Collins, CO, USA) and representative sugar beet from greenhouse experiment harvest.



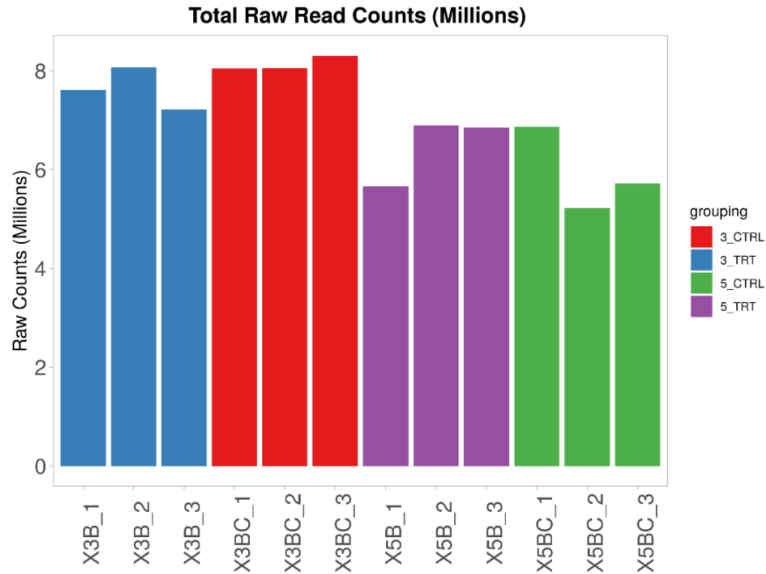
Supplementary Figure 2.6 Serial dilution of C53, a consortia of *Brevibacillus* sp. P2.F4.A, *Bacillus cereus* 4B.1B.PK and *Serratia marcescens* 10A.1A.P216 at a (A) 10^{-3} and (B) 10^{-5} dilution.



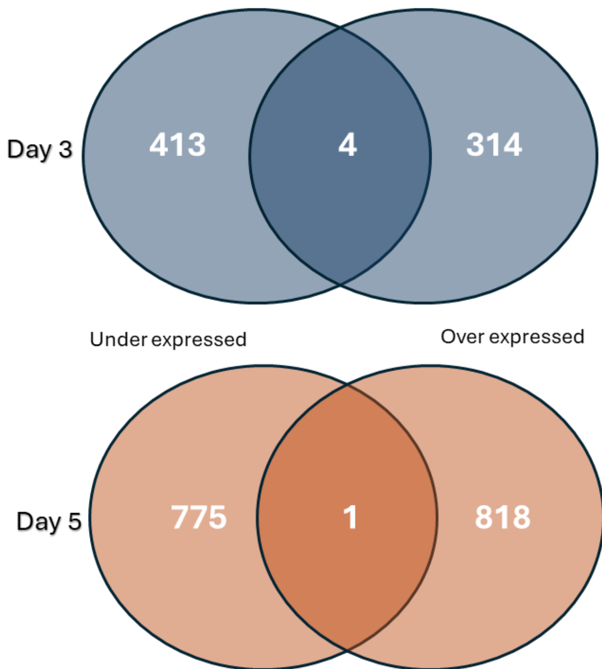
Supplementary Figure 2.7 Gene abundance in soil samples collected immediately before sowing seeds for damping off experiment. Figures are based on normalized Ct values from qPCR data with (A) *Rhizoctonia solani* AG2-2 and (B) total bacterial primers.



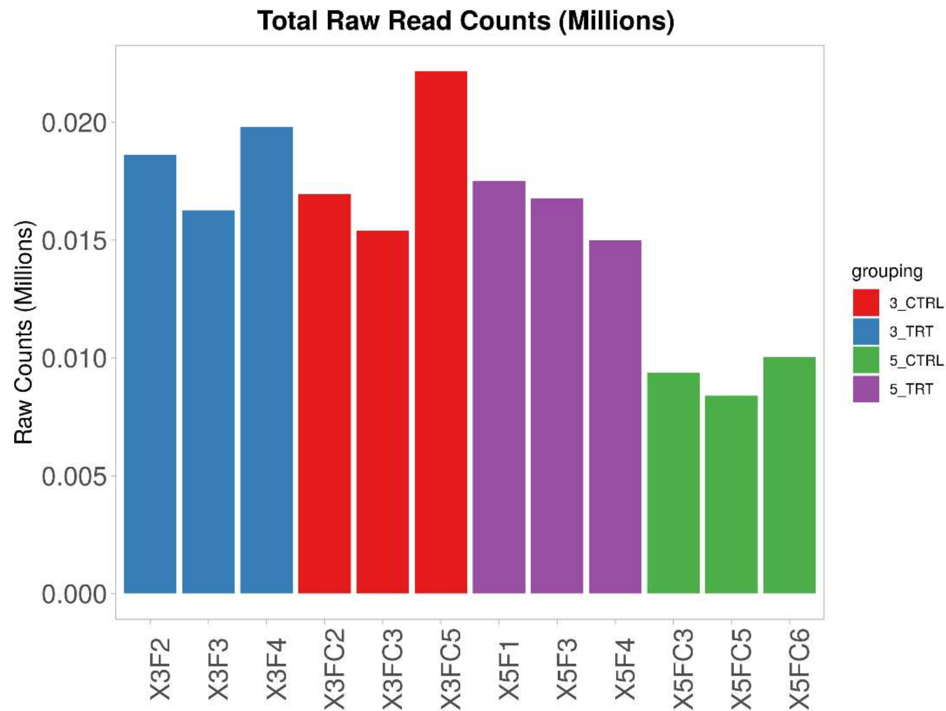
Supplementary Figure 2.8 Gene abundance in soil samples collected immediately before transplanting 5-week-old sugar beet plants for the mature root and crown rot experiment. Figures are based on normalized Ct values from qPCR data with (A) *Rhizoctonia solani* AG2-2 and (B) total bacterial primers.



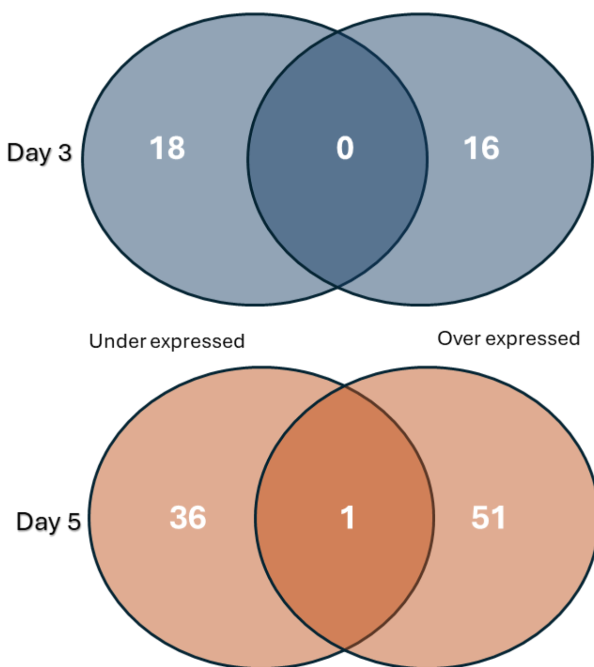
Supplementary Figure 3.1 Total raw read counts for bacterial samples.



Supplementary Figure 3.2 Total differentially regulated gene counts for bacterial samples at day 3 (blue) and day 5 (orange). Under expressed genes are on the left, both under and over expressed in the center, and over expressed on the right.



Supplementary Figure 3.3 Total raw read counts for fungal samples.



Supplementary Figure 3.4 Total differentially regulated gene counts for fungal samples at day 3 (blue) and day 5 (orange). Under expressed genes are on the left, both under and over expressed in the center, and over expressed on the right.