BRASSICA COVER CROPS ALTER WEED CO-OCCURENCES AND SOIL MICROBIAL COMPOSITION

A Thesis

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ABSTRACT

Farmers utilize cover crops to manage weeds, amend nutrients, improve resiliency against pest and disease, and build soil health. One limitation to adoption is that we cannot predict well enough how different cover crop species affect weed management or soil health. Brassica cover crops are a well-studied group because of their potential for agriculturally and ecologically significant weed control. The functions of soil microbiomes could contribute to underlying mechanisms behind these weed responses. Two properties of brassica cover crops have substantial and differing effects on the soil microbiome and weed community: glucosinolate and carbon release through root exudation and biomass breakdown. Glucosinolates break down into biocidal isothiocyanates that have been found to effectively control weeds and soil pathogens. Carbon from cover crop biomass and root exudates also stimulate microbial activity and create more diverse resource pools, resulting in more diverse weed and microbial communities. This thesis assesses whether the soil microbiome differed under cover crop treatments (Chapter 1), and if differing weed and microbial responses could distinguish between carbon and glucosinolate as driving mechanisms (Chapter 2).

BIOGRAPHICAL SKETCH

Jenny Berkowitz was born in 1996 in Minnesota. She has a younger sister, Mary Walz, and her parents are Robert and Kathy Walz. Jenny met her husband, Adam Berkowitz, while running on the cross country team during college. Jenny graduated Summa Cum Laude from the University of St. Thomas in St. Paul, Minnesota with a Bachelor of Science in Biology and Bachelor of Arts in Environmental studies in 2018. After working in agriculture for a year, she decided to continue her education in the Department of Horticulture at Cornell University.

To my husband, who traveled across the country to be my constant support. You keep me grounded.

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To Jenny and Thomas, for pushing me to accept the flaws of my research and write about it anyways. You created a path for my career.

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LIST OF ABBREVIATIONS

16S	16S rRNA
ANOVA	Analysis of variance
AMF	Arbuscular mycorrhizae fungi
ASV	Amplicon sequence variants
ITC	Icothiocyanates
ITS	ITS2 Region of internal transcribed spacer gene
Maaslin2	Microbiome multivariable association with linear models 2.0
MSM	Mustard seed mal
NMDS	Non-metric multidimensional scaling
OTU	Operational taxonomic unit
PCoA	Principal Coordinates Analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
PSFs	Plant-soil feedbacks
QIIME2	Quantitative insights into microbial ecology 2
RCBD	Randomized complete block design

CHAPTER ONE

Weed community co-occurrences and rhizosphere microbiomes are influenced by Brassica cover crops

Abstract

Farmers utilize cover crops to manage weeds, amend nutrients, improve resiliency against pest and disease, and build soil health. One limitation to adoption is that we cannot predict well enough how different cover crop species affect weed management or soil health. Two properties of cover crops have substantial and differing effects on the soil microbiome and weed community: glucosinolate and carbon release through root exudation and biomass breakdown. We assessed these properties using two fall planted crucifer cover crops: *Brassica hirta*, cv. 'Tilney' (yellow mustard), and *Raphanus sativus L* (forage radish). We hypothesized that Brassica cover crops reduce weed abundance while enhancing weed species diversity. Additionally, we hypothesized that we would observe ecological responses to carbon influx and glucosinolate allelopathy. Weed analyses using ANOVA, PERMANOVA and indicator species exhibit similar trends: the control treatment promoted warm season and nitrophilous weeds like Pigweed, Lambsquarters and Purslane, while the cover crop treatments select for cool season weeds like henbit and exhibit a more diverse weed population. Fungal and bacterial community compositions were significantly different between all three treatments from PERMANOVA, PCoA and hierarchical clustering analyses. Weed responses were consistent with carbon ecological responses, with some patterns consistent with cover crop plant residue effects. Taxonomic promotion or repression of certain fungal and bacterial OTUs, according to Maaslin2, showcase both carbon and glucosinolate ecological theories with both decreases and increases in some pathogenic and nitrogen fixing bacteria and fungi. More research is necessary to better understand the confounded carbon and glucosinolate mechanisms involved behind brassica cover crop weed control.

Introduction

Farmers utilize cover crops to manage weeds, amend nutrients, improve resiliency against pest and disease, and build soil health. Different species of cover crops are better suited

for each of these goals, and responses can depend on different soil types, farm management, and cropping systems. One limitation to adoption is that we cannot predict well enough how different cover crop species affect weed management or soil health. In particular, we do not know the extent to which different mechanisms such as nutrient competition or root-soil interactions control cover crop agronomic effects. Two properties of cover crops have substantial and differing effects on the soil microbiome: metabolite and carbon release through root exudation and biomass breakdown. These properties are highly confounded, but each have important ecological effects that drive weed and microbial community composition.

Cover crops are a sustainable complement to weed management practices like tillage and herbicides. Tillage can be damaging to soil health, defined by the US Department of Agriculture as the "continued capacity of soil to function as a vital living ecosystem that sustains plants, animals, and humans" (https://www.nrcs.usda.gov/wps/portal/nrcs/main/soils/health/). Reducing tillage is a common strategy for promoting greater soil health (Nunes et al., 2020) because tillage disrupts important soil aggregating properties that enhance carbon sequestration, improve water-holding capacity, and prevent soil erosion (Six et al., 1999). Forage radish act as an alternative to tillage through biological chiseling, or physically creating holes in the soil due to large root growth. Compared to conventional tillage, forage radish has been found to improve soil aggregation, crop root development, and soil organic carbon stocks (Guedes Filho et al., 2013; Inagaki et al., 2021). Herbicides could also be damaging to soil health through unpredictable harmful impacts on soil microorganisms (Bhardwaj et al., 2021), though farmers are more often choosing to reduce reliance on herbicides due to weed resistance or difficulty accessing appropriate herbicides (Peterson et al., 2018).

Cover crops are important tools for alternative weed management that benefit soil health by stimulating soil microorganisms, increasing soil organic matter, and fixing nitrogen (Honeycutt et al., 2020; Magdoff, 2001; Nivelle et al., 2016; Ranaivoson et al., 2017). Vegetable production is of interest for soil health because it relies on more tillage, and returns less biomass than field crop systems, making soil-health goals more difficult to meet. Vegetable producers also have fewer herbicides available to them for weed control, so cover cropping can be a valuable weed management tool (Fennimore and Doohan, 2008).

The two crucifer cover crops, yellow mustard (*Brassica hirta*, cv. 'Tilney') and forage radish (*Raphanus sativus L*) each uniquely impact soil health and weed control. Many Brassica roots produce a substantial amount of glucosinolates (sinalbin in Tilney), located in the outer cells of the root (McCully et al., 2008) or the seed (Gamba et al., 2021) and release biocidal isothiocyanate (*p*-hydroxybenzyl isothiocyanate in Tilney) if damaged (Tsunoda et al., 2018). This biocidal effect is used in biofumigation in other climates where large mustard biomass is obtained shortly before planting (Fouché et al., 2016; Hoagland et al., 2008; Kirkegaard and Sarwar, 1998). Biofumigation can selectively kill certain microorganisms and weeds, with varying downstream applications for soil health conservation and weed control.

Cover crops release metabolites that impact soil microbial communities in varying ways. Glucosinolate is one of the most researched metabolites that plants use as a chemical defense. Isothiocyanates (ITC) are generally identified as antifungal, especially pathogens such as *Rhizoctonia solani, Sclerotinia sclerotiorum, Alternaria brassicicola,* and *Fusarium oxysporum* (Plaszkó et al., 2021). Studies have found significant decreases in overall fungal abundance after exposure to glucosinolate compounds and brassica plant biomass (Hollister et al., 2013; Plaszkó et al., 2021). Glucosinolates also increase bacteria with antifungal properties such as *Streptomyces* (Hollister et al., 2013), or bacterial plant pathogens such as *Pythium spp.* (Brown and Morra, 1996; Hoagland et al., 2008; Jin et al., 2019).

Other studies have found significant weed suppression associated with high glucosinolate content seed meals or plant residues (Earlywine et al., 2010). Glucosinolates have been found to reduce Pigweed, Shepherd's Purse, Kochia (*Bassia scoparia*), Wild Oat (*Avena fatua*), Italian Ryegrass (*Lolium multiflorum*), and Prickly lettuce (*Lactuca serriola*), among many others (Handiseni et al., 2011; Krishnan et al., 1998; Singh et al., 2006). Forage radish and other brassica plants specifically reduce early spring weed germination. The studies attribute these weed reductions to light and nutrient competition or reduced weed seed fall in the fall rather than glucosinolate (Gieske et al., 2016; Lawley et al., 2012). Observing significant decreases in fungal diversity, pathogens, and spring weeds could help identify if glucosinolate allelopathy is occurring.

Cover crop roots and biomass add carbon into the soil which alters soil microbial community composition and activity (Kuo et al., 1997). Carbon amendments have been found

to decrease *Acidobacteria* and increase *Bacteroidetes* and *β-Proteobacteria* (Fierer et al., 2007). Plant carbon secretion changes the soil microbial community in the rhizosphere by providing a food source for microbiota, this in turn is associated with increased microorganism abundance (27%), activity (22%), and diversity (2.5%) according to a recent meta-analysis (Kim et al., 2020). As carbon driven microbial activity increases, microorganisms immobilize nitrogen within their bodies, reducing nitrogen availability to plants (Mooshammer et al., 2014). This resultant nitrogen immobilization can select against nitrophilous weeds such as amaranths (Gannett et al., 2022). If carbon addition plays a role in weed and microbial community co-occurrence, we expect to see reduced nitrophilous weeds due to their demands for inorganic nitrogen to support rapid growth.

Cover crop nutrient amendments also create more diverse resource pools. According to the resource pool hypothesis (Smith et al., 2010), a more diverse resource pool encourages growth of diverse weeds that can take advantage of the different nutrients in the soil. This leads to decreased weed-crop competition for certain nutrients as different growth strategies would be favored (MacLaren et al., 2020; Storkey and Neve, 2018). Soil microbial diversity may be enhanced when plant diversity increases, as shown in several empirical studies (Carney and Matson, 2006; Venter et al., 2016; Vukicevich et al., 2016) The changes in soil nutrient pool from cover crops could increase both weed and microbial community diversity.

Although cover crop metabolite and carbon exudation interactions are complex and difficult to measure in field settings, we can examine the weed and microbial community responses that differ between two cover crops that produce glucosinolates versus a control that has no plant cover treatment. We assessed these properties using two fall planted crucifer cover crops: *Brassica hirta*, cv. 'Tilney' (yellow mustard), and *Raphanus sativus L* (forage radish). These cover crops have distinctive soil affects that affect the soil microbiome in their valued soil health niche as a fall sown cover crop in vegetable production. We surveyed soil bacterial and fungal populations in the spring along with weed germination in the spring, post maize plant, and in the summer.

In many cases, Brassica cover crops reduce weed abundance. We hypothesized the cover crops would either selectively suppress certain weed species more than others or affect weed species equally. The soil microbiome could be an underlying mechanism behind weed

responses, if that is the case, the soil microbiome would be different under cover crop treatments. We hypothesized there would be different soil bacterial and fungal community composition under cover crop treatments, or no difference would negate the role of the soil microbiome in weed suppression. Nuanced weed and microbial community responses may be explained by ecological responses to carbon and glucosinolate.

Materials and Methods

Radish and mustard cover crops were planted in Fall 2019. All data was collected the following growing season. The field was maintained following standard maize farming practices in a reduced tillage system. We surveyed weed germination after each cultivation at key ecological timepoints: spring, post-plant, and summer. Cover crop residue was cultivated after the spring weed survey, residue pictures are available in the supplemental figures (**Figure 11 and 12**). Soil samples for bacterial and fungal community composition were collected at the spring timepoint before any cultivation.

Treatment blocks were created in the Cornell AgriTech Field Complex in Geneva, NY in September 2019. There were two blocks each of mustard, radish, and control, separated into a total of 24 experimental units, eight each.

The three weed surveys were conducted on May 26th, June 23rd, and July 7th. Weed surveys were conducted after cultivation as soon as weed species were identifiable. We identified and recorded the number of each species within 4, 1 m^2 segments for each experimental unit. The 1 m^2 plots were placed in the same location each weed survey and averaged over each experimental unit to account for field variation. Upon completion of the weed survey, weeds were cultivated again, and the weed survey cycle was repeated until cultivation was no longer possible due to maize height.

All weed survey statistical analysis was conducted in R version 4.1.2 (R core team, 2022). Weed total abundance, richness, evenness, and Shannon diversity indices were calculated for each weed survey. Weed total abundance is the sum of all individuals found in a sample. Richness is the sum of all species in the sample. Evenness is the Shannon diversity index divided by the log of richness. Shannon diversity was calculated (function diversity, in package vegan, version 2.5-7). All four were analyzed using analysis of variance (ANOVA)

followed by Tukey HSD to assess differences between treatments. Percent weed change from control due to mustard and radish treatments was calculated for each weed species [(new-control)/control*100], then analyzed using ANOVA and Tukey post-hoc on log transformed data. Percent change from control to cover crop, and between the mustard and radish cover crops was also calculated and analyzed the same way. Weed community composition differences were assessed on Wisconsin and square root transformed Bray-Curtis distances (Holland, 2008) using Permutational Multivariate Analysis of Variance (PERMANOVA) with 999 permutations (function adonis, in package vegan, version 2.5-7) and Non-metric multidimensional scaling (NMDS, function mds, in package vegan, version 2.5-7). Indicator species were identified from the weed community reported with indicator values and correlation values (indicspecies package, version 1.6.7).

Soil samples for bacterial and fungal community composition were collected on May 26th. Eight, 6" deep soil cores were taken for each experimental unit using a 22 mm wide soil push probe (Classic soil probe; Oakfield apparatus company, Oakfield, WI) and aggregated into one sample to account for field variation. Samples were stored in a cooler in the field and then placed in a refrigerator set to 4°C until processed.

DNA extractions were conducted from approximately 2.5 g of soil using the Qiagen DNeasy extraction kit (Beverly, MA) using the provided instructions. We used high-throughput sequencing to assess the V3-V4 region of bacteria, 16S rRNA (16S), and fungi, ITS2 region of internal transcribed spacer gene (ITS). DNA was amplified by running Polymerase Chain Reactions (PCR) on a Bio-Rad C1000 Thermal Cycler (Hercules, CA). Reactions used 8 µL of 5 PRIME HotMasterMix (5 PRIME Inc, Gaitherburg, MD), 1 µL of both forward and reverse primers for the 16S or ITS regions, respectively, 0.5 µL of BSA for 16S regions, 1 µL of DMSO for ITS regions, and sterile water for a total volume of 20 µL.

Amplicons were cleaned using MagBio HighPrep PCR beads (MagBio Genomics, Gaithersburg, MD) and amended with unique barcode index primers. Labeled amplicons were then pooled, concentrated (Centri-vap DNA concentrator; Labconco, Kansas City, Missouri), and additionally cleaned (Wizard SVG gel and PCR clean-up system; Promega, Madison, Wisconsin). Samples were then sequenced on the Ilumina MiSeq at the Cornell Genomics Facility (Ithaca, NY). Raw sequence reads were prepared in Quantitative Insights Into

Microbial Ecology 2 (Qiime2) (Bolyen et al., 2019) denoised into amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016), and clustered into operational taxonomic units (OTUs) (Edgar et al., 2011). OTUs were then assigned taxonomy based on a Naive Bayes Classifier trained on the UNITE_ver8_dynamic database for ITS and on the 99% greengenes database 13_8 for 16s (Abarenkov et al., 2010; DeSantis et al., 2006).

All further microbial analyses were conducted in R version 4.1.2 (R core team, 2022). Bacterial and fungal samples were rarified (randomly sample a set number of reads) to match the minimum reads of OTUs present (rarify function, package vegan, version 2.5-7). Bacterial and fungal community richness, evenness and Shannon Diversity were calculated on OTU percent abundance [(OTU/Total OTUs)*100], and analyzed for treatment effects using ANOVA and Tukey post-hoc test. A PERMANOVA using 999 permutations was run on the Bray-Curtis distances to quantify statistical differences between treatments. Then, Principal Coordinates Analysis (PCoA) was conducted to assess community differences. Cluster analysis using Wards method was used to validate PCoA groupings (Landau and Chis Ster, 2010). We used the machine learning algorithm randomForest (fuction *randomForest*, in package randomForest, version 4.6-14) to predict the treatment based off OTU percent abundance with or without rare genera removed. This algorithm then displays a subset of OTUs that are most predictive for each treatment. The function Microbiome Multivariable Association with Linear Models 2.0 (Maaslin2) was also used to identify OTUs associated with each treatment. Maaslin2 conducts linear and mixed models to compare the percent abundance of each OTU between treatments. It then identifies OTUs that have significant differences, either present or not present, in each treatment and adjusts for multiple hypothesis testing (Mallick et al., 2021). Rather than a predictive decision tree algorithm like randomForest, Maaslin2 begins with treatment groups and then identifies OTUs that are promoted or repressed in a treatment.

Results

Weed Community Composition

Cover crop treatments increased weed total abundance in the spring weed survey ($p < 0.001$), but then reduced weed total abundance by 96.8% and 89.9% for the post plant and summer surveys respectively ($p < 0.01$, $p < 0.001$). Weed species richness, or the number of

species present in a sample, was only statistically different in the spring weed survey, where radish displayed higher species richness than mustard ($p < 0.01$). Weed species evenness was significantly higher in the mustard treatment compared to control in the spring weed survey ($p = 0.002$), and higher in both cover crop treatments in the second two weed surveys ($p < 0.001$, $p<0.001$). Shannon diversity (**Figure 1**) followed a similar pattern to weed total abundance, with different responses in the spring survey compared to the following two survey dates. Throughout the season, the diversity of weed species in the radish and mustard cover crop treatments remained the same, while the control significantly decreased after the spring weed survey. Mustard exhibited greater species diversity, as indicated by the higher Shannon diversity index, in the spring survey compared to the radish and control treatments. The radish cover crop did not show responses similar to the mustard treatment ($p < 0.01$). Both cover crops showed increased Shannon diversity by 110.0% and 59.5% in the post plant and summer weed surveys respectively ($p < 0.01$, $p < 0.001$). The significantly lower diversity in the control is due to the domination of certain weed species: *Amaranthus* sp. (pigweed), *Chenopodium album* (lambsquarters), and *Portulaca oleracea* (purslane).

Weed species were affected differently by the cover crops and throughout the field season, weed species reductions due to cover crops are shown in **Table 1**. Mustard or both cover crops consistently reduced pigweed across all three weed surveys, and reduced purslane, and lambsquarters in the post plant and summer weed surveys. Unknown broadleaf weeds and *Lamium amplexicaule* (henbit) increased in mustard, radish, and cover crop treatments in all three weed surveys. Weed community composition, as indicated by NMDS and confirmed by PERMANOVA (**Figure 2)**, indicate significantly different weed communities between bare, mustard and radish treatments in all three weed surveys ($p < 0.001$ for all).

Indicator species represent a certain treatment due to their abundance or affiliation with diversity of other species within each weed community (Caceres, 2013). Species are reported with indicator values that signify the degree that a certain species can be used to predict a treatment, and correlation values that identify the ecological preferences of a different weed species (Caceres, 2013). Indicator species were identified for each weed survey (**Figure 2**). In the spring weed survey, Pigweed was an indicator species for control and radish ($p < 0.01$), while Chickweed was an indicator species for radish and mustard ($p < 0.01$). The same

relationships were reported in the correlation values. In the post plant weed survey, lambsquarters, pigweed and purslane were indicator species for the control ($p < 0.01$ for all), and Henbit was an indicator species for radish and mustard ($p < 0.01$). Correlation values only identified purslane as significantly correlated with the control ($p < 0.05$). In the summer weed survey, pigweed was an indicator species for control ($p < 0.05$), lambsquarters was an indicator for control and radish ($p < 0.01$), and unknown broadleaf weeds and henbit were indicators for both cover crops ($p < 0.01$, $p < 0.05$). The ANOVA, PERMANOVA and indicator species analyses exhibit supporting trends: control tends to select for warm season and nitrophilic weeds like pigweed, lambsquarters and purslane, while the cover crops select for cool season weeds like henbit and exhibit a more diverse weed population.

Microbial Community Composition

In total, 1,358 fungal sequences and 4,639 bacterial were observed in the 23 soil samples. There was a mean of 29,756 reads per fungal sample and 15,275 reads per bacterial sample. One sample from control was removed from bacterial and fungal datasets due to low reads. Fungal species alpha diversity and richness in the radish treatment were decreased by 8.3% and 14.8% respectively ($p = 0.03$, $p = 0.01$), richness was decreased in the radish treatment only (14.8%, $p = 0.01$), evenness was not different between treatments (**Figure 3**). Mustard Shannon Diversity was the same as the control, and higher than the radish treatment ($p = 0.99$, $p = 0.03$), richness followed the same pattern ($p = 0.56$, $p < 0.01$), evenness was not affected. Similarly, bacterial alpha diversity and species richness were decreased by 5.8% and 28.6% respectively ($p = 0.03$, $p = 0.03$), and evenness increased by 6.4 % in the radish treatment ($p = 0.03$) compared to control (**Figure 4**). The mustard treatment did not have different Shannon Diversity, richness, or evenness compared to both the control and radish treatment ($p = 0.36$, $p = 0.34$; $p = 0.29$, $p = 0.53$; $p = 0.5$, $p = 0.29$).

The composition of fungi in the samples clearly differentiated among control, radish, and mustard treatments in the PCoA analysis (**Figure 5**), with PERMANOVA supporting the differences ($p = 0.001$), and equivalent dispersion ($p = 0.37$). Bacterial composition demonstrated a clear separation in the PCoA based on the presence or absence of cover crops, with some differences between mustard and radish (**Figure 6**). The PERMANOVA showed

significant differences between the presence and absence of cover crops ($p = 0.001$), distinguishing between radish and mustard cover crops ($p = 0.037$). The control, which has a slightly smaller sample size, exhibited a lower dispersion than the radish and mustard treatments ($p = 0.003$), and so the PERMANOVA is likely overly conservative (Anderson and Walsh, 2013). However, the PERMANOVA was significant, and the dispersion differences are not an issue. Cluster analysis for both fungal and bacterial datasets, displayed in relative abundance heatmaps (**Figure 13 and 14**), identified three distinct clusters. The control samples were in one cluster, while mustard and radish were spread across two other clusters. The cluster analysis mirrors the patterns displayed in the PCoA microbiome ordination groupings.

The machine learning algorithm, randomForest, conducts a similar process to the Indicator species test used in the weed analysis by randomly creating decision trees based off of microbial taxa, or OTUs in this analysis. The decision trees create a model based on a subset of data that can predict a treatment based on the predictor OTUs. The model is validated utilizing the entire data set, and percent error is reported. Generally, models exhibited higher accuracy when rare genera were removed. RandomForest created models for the fungal dataset that had error rates that ranged as low as 17% up to 38% error. The randomForest models for the bacterial data set were even more inaccurate with between 26% error and 38% error. This means that the model incorrectly predicted the treatment between 26 and 38% of the time, suggesting the inability to properly identify meaningful OTUs and a risk of overfitting the model (Hastie et al., 2009). Though the most accurate randomForest models did identify predictor OTUs for both bacterial and fungal datasets, the error rate is too high to support further investigation.

Maaslin2 is more effective than randomForest for use with datasets that have nuanced OTU differences between treatments. Unlike randomForest, which relies on identifying more important or predictive OTUs, Maaslin2 conducts t-tests on every OTU and reports the treatment differences for each. Two processes were applied to each bacterial and fungal dataset, first identifying differences between presence or absence of cover crops, then between mustard and radish. The top 20 fungal OTUs that are most significantly different between presence or absence of cover crops are listed in **Table 2**. These OTUs were split by a distance matrix, with samples that are most different from each other placed into two groups (**Figure 7**). The groups

aligned by cover crop and no cover treatments, confirming the ability of Maaslin2 identified OTUs to distinguish different treatments. The top 20 fungal OTUs that are significantly different between mustard and radish cover crop treatments can be found in **Table 3**. Fungi also clustered well between radish and mustard treatments (**Figure 8**). Similarly, bacterial OTUs between presence or absence of cover crops and between mustard and radish are listed in **Table 4** and **Table 5** respectively. Both distance matrices perfectly grouped the samples by treatment, which confirms effective separation of significant OTUs (**Figures 9 and 10**).

Problematic fungal pathogens and other fungal genera that are commonly suppressed by glucosinolate were also assessed using ANOVA and Tukey post-hoc tests. Genera identified in literature that were present in the fungal dataset include *Ascobolus*, *Chaetomium*, *Humicola*, *Fusarium*, and *Mortierella* (Brown and Morra, 1996; Hoagland et al., 2008; Jin et al., 2019; Plaszkó et al., 2021). Only *Mortierella* was significantly suppressed by mustard and radish treatments ($p = 0.006$, $p < 0.001$ respectively). The top 20 significantly different OTUs from Maaslin2 supports that cover crops suppress the genus *Mortierella* and suppresses almost all the rest of the OTUs identified except genera *Pyrenochaetopsis*, *Syncephalis*, and *Ganoderma*. *Aspergillus clavatus*, *Staphylotricum coccosporum*, *Occultifu mephitis*, and *Aspergillus clavatus*. Radish and mustard treatments did show some interesting abundance patterns, with mustard suppressing genera *Alternaria*, *Mortierella*, and *Neonectria* more significantly than radish. On the other hand, radish suppressed *Fusarium*, and *Penicillium* more than mustard.

Bacteria taxon responses to carbon in literature such as decreased *Acidobacteria* and increased *Bacteroidetes* and *β-Proteobacteria* populations were assessed using ANOVA and Tukey post-hoc tests of relative abundances. There were no significant changes in these populations, except *Acidobacteria* was significantly lower in radish compared to mustard ($p = 0.03$). Maaslin2 identified several additional OTU suppression or promotion, most importantly it confirmed that cover crops suppressed *Acidobacteria*, and promoted *Solirubrobacterales*, and *WD2101*. Though there were different bacterial OTUs between mustard and radish, abundance figures do not demonstrate any notable OTUs.

Discussion

Weed community composition under Brassica cover crops

Brassica cover crops often reduce weed abundance. We did see weed reduction, though like we hypothesized, certain weeds were selectively suppressed more than others. Weeds were more diverse under the cover crop treatment, and the cover crops reduced pigweed, lambsquarters, and purslane the most. These three weeds are agronomically important and can be particularly problematic for farmers due to their competitiveness and herbicide resistance.

Cover crop treatments affected certain weed community diversity and species dynamics in patterns consistent with the resource pool hypothesis and nitrogen immobilization. According to the resource pool hypothesis, a more diverse pool of resources leads to diverse weeds due to different growth strategies (MacLaren et al., 2020; Storkey and Neve, 2018). Our results model this pattern with increased Shannon diversity indices in the cover crop treatments during the post-plant and summer weed surveys. Higher diversity in the weed community inversely correlates with weed-crop competition. Weed diversity in the spring weed survey likely did not differ due to an overall high diversity of cold season weed species in all treatments. Increased weed diversity was present during the most agronomically important time points during crop establishment. This factor is particularly useful for growers that struggle with one or two dominant weed species that are responsible for most of the crop loss.

Weed community co-occurrences follow patterns that suggest nitrogen immobilization in the soil. Ecological studies have identified carbon influx into the soil, in our case from cover crop root exudates and biomass, stimulates soil microbial activity. To match increased carbon intake, microorganisms immobilize nitrogen within their bodies, reducing nitrogen availability to plants (Little et al., 2021; Mooshammer et al., 2014). This resultant nitrogen immobilization can select against weeds that require high nitrogen soils for their physiological processes (Gannett et al., 2022). These weeds, called nitrophilous weeds, co-occurred in the control treatment, but were suppressed by cover crops. Pigweed was significantly reduced in mustard and radish treatments in all three weed surveys, and lambsquarters and purslane were reduced by the cover crop treatments in the second two surveys. Pigweed, lambsquarters and purslane all thrive in high nitrogen environments (Abouziena et al., 2007; Costea et al., 2004; Jäck et al., 2021) and so are susceptible to nitrogen immobilization that may occur with the cover crop

driven carbon increases in the soil. Varied lambsquarters responses over time and between different cover crop treatments may be caused by its physiological ability to adapt to low nitrogen environments better than Pigweed, but still worse than other non-nitrophilous weeds (Sage and Pearcy, 1987). Despite the lower response in the spring weed survey, Lamb's Quarter and Pigweed reduction occurred at the most ecologically important timepoints, immediately after crop planting and throughout the summer. Improved pigweed control is important for farmers because it is particularly problematic for crop yield and glyphosate resistance (Sosnoskie et al., 2009; Webster and Nichols, 2012). Purslane and Lamb's Quarter are also common weeds that can significantly reduce crop yield, especially in corn and vegetable systems (Miyanishi and Cavers, 1980; Sosnoskie et al., 2009).

Glucosinolate allelopathy could also play a role in weed co-occurrences (Handiseni et al., 2011; Krishnan et al., 1998; Singh et al., 2006). Some studies reported brassica cover crops reduced early spring weeds (Gieske et al., 2016; Lawley et al., 2012), though they hypothesized the driving mechanism behind this response was light competition. Contrary to these findings, our study found that radish and mustard cover crops increased henbit and some other broadleaf and winter weeds in the spring weed survey and throughout the season. This may be due to cover crop driven changes in soil temperature or soil moisture. Cover crop residue, which was still present during the spring weed survey (**Figure 11 and 12**), can maintain lower soil temperatures and higher soil moistures (Munawar et al., 1990; Wagner-Riddle et al., 1994; Zibilske and Makus, 2009). This creates a more hospitable environment for winter weeds. Radish had lower ground coverage than the mustard treatment (**Figure 11 and 12**), if cover crop residue were a major mechanism, we would have expected mustard to exhibit higher weed abundance than radish. On the other hand, increases in henbit, chickweed and other broadleaf weeds were often better controlled by the radish treatment than the mustard. It is not clear if cover crop physical effects are responsible for the increased winter weeds, and more research is necessary to determine the mechanisms behind winter weed promotion.

Microbial community composition is different under brassica cover crops

Both bacterial and fungal communities were significantly different between all three treatments, though ecological and agricultural implications of these differences are complex

and understudied. Cover crop treatments affected certain microorganism species in patterns consistent with expected responses from both carbon and glucosinolate mechanisms.

Fungal Community Differences

The cover crop treatments substantially changed fungal taxonomic responses. Out of the 20 Maaslin2 identified OTUs, 11 were enriched in the control, while nine were enriched in the cover crops (**Figure 7**), several of these OTUs were either taxonomically unidentifiable or their function was unknown (Yurkov, 2018). Fungal diversity and richness were decreased by the radish cover crop. Since soil samples were collected at the same time as the spring weed survey, the decreased Shannon Diversity correlates with the decreased weed diversity at that time. This correlation is consistent with the resource pool hypothesis and ecological studies that have found interactions between plant and microbial diversity (Carney and Matson, 2006; Venter et al., 2016; Vukicevich et al., 2016). Our data does not provide enough evidence for overall fungal suppression, future research should measure fungal abundance and activity as an additional metric.

The OTUs enriched in the control include: *Acremonium persicinum*, known to be antimicrobial (Nakamura et al., 2017); *Papiliotrema laurentii*, associated with root exudates and arbuscular mycorrhizae fungi (AMF) (Sarkar et al., 2019), *Occultifur mephitis*, prefers lower carbon soils (Šibanc et al., 2018); *Mortierella*, may increase nutrient uptake efficiency (Ozimek and Hanaka, 2021); and *Staphylotrichum coccosporum*, from the family *Sordariales* connected to secondary metabolite production (Charria-Girón et al., 2022). The absence of these genera in the cover crop treatment provides mixed messages about the underlying ecological mechansims. If glucosinolate allelopathy was occurring, we would have expected more (not less) antimicrobial fungi such as *Acremonium persicinum* in the cover crop treatments, while the absence of the AMF associated *Papiliotrema laurentii* supports a brassica specific response since brassicas do not form relationships with AMF. Glucosinolate allelopathy often reduces plant-pathogenic fungi. The suppression of *Mortierella* was consistent with glucosinolate responses in Plaszkó et al., 2021. Contrary to glucosinolate alleopathic studies, three pathogenic OTUs were enriched in the cover crop treatment, *Pyrenochaetopsis* produces some metabolites similar to pathogen Fusarium (Fan et al., 2020); *Syncephalis* is a

mycoparasite specific to apricot trees (Jeger and Spence, 2001; Lazarus et al., 2017), and *Ganoderma* is used medicinally for its anti-microbial effects (Paterson, 2006). The increase in anti-microbial *Ganoderma* supports our glucosinolate hypothesis but increases in potentially pathogenic genera such as *Pyrenochaetopsis* and *Syncephalis* directly negate our expected glucosinolate responses. Ecologically, we would expect increases in carbon due to cover crops to select against organisms that are more competitive in low carbon and low nutrient soils. This was observed in our cover crop treatment with decreases in *Occultifur mephitis* and *Mortierella*.

Interestingly, Maaslin2 identified some noteworthy OTU differences between radish and mustard treatments. Mustard enriched several genera associated with pathogens such as *Alternaria* (Tralamazza et al., 2018) and *Fusarium* (Mesny et al., 2021). These differences in OTU abundance (**Figure 8**), suggest different fungal dynamics between radish and mustard cover crops that are worth further investigation.

Bacterial Community Differences

Bacteria taxonomic changes match some of the expected carbon influx responses. Similar to the (Fierer et al., 2007) meta-analysis, we observed the repression of *Acidobacteria* in cover crop treatments, which have been identified to only selectively digest certain forms of carbon (Kielak et al., 2016) potentially making them less competitive in a high C:N soil environment. Maaslin2 also identified *Solirubrobacterale* to be associated with cover crop treatments. This order of bacteria is commonly found alongside *Acidobacteria,* but little is known about its ecological function (Shange et al., 2012; Williams et al., 2016). Maaslin2 identified cover crops promoted the order *WD210,* which degrade polysaccharides in peatlands (Dedysh et al., 2020), making this order an ideal candidate to take advantage of cover crop carbon additions. On the other hand, we did not see any impact on *Bacteroidetes* and *β-Proteobacteria* as shown in Fierer, 2017. Unlike the Kim et al., 2020 meta-analysis, we observed little to no impact on bacterial diversity, in fact radish treatments exhibited marginally lower diversity.

If glucosinolate allelopathy played a role in bacterial community differences, we expected cover crops would dramatically decrease bacteria with antifungal properties like *Streptomyces* (Hollister et al., 2013), or increased bacterial plant pathogens such as *Pythium*

spp. (Brown and Morra, 1996; Hoagland et al., 2008; Jin et al., 2019). Neither of these groups were present in the bacterial community data set, and therefore we were unable to support or disprove if cover crops did suppress these groups.

Conclusions

Consistent with our hypothesis, cover crops did selectively affect certain weed species. Mustard and radish cover crops reduced pigweed, lambsquarters and purslane by an average of 89%, 64%, and 89% respectively. These weeds are particularly problematic in vegetable systems in the U.S. and so controlling them with mustard cover crops could help reduce negative impacts on crop yield. Pigweed, lambsquarters and purslane are all nitrophilous weeds, therefore carbon may be an important mechanism behind brassica cover crop weed control due to stimulated microbial activity that immobilizes nitrogen. The cover crops also significantly affected the soil microbiome, confirming that the microbiome may be an underlying mechanism for weed suppression. Radish treatments decreased fungal and bacterial diversity measurements. Taxonomic analysis of both fungal and bacterial datasets could be explained by carbon and glucosinolate ecological theories, with some nutrient hungry microorganisms decreased by cover crops, and some pathogenic microorganisms reduced. It is not clear that there were any changes to nitrogen fixing microorganisms, which would have been expected if carbon influx was stimulating specialists that are well adapted for high soil C:N ratio.

Though this study utilizes weed and microbial community responses to improve our understanding of the mechanisms behind cover crop driven weed suppression, carbon and glucosinolate are highly confounded. In addition to carbon and glucosinolate, there are many other ecological responses to cover crops that could drive weed suppression. More research aiming to specifically study the ecological responses to carbon, glucosinolate, and cover crops separately is needed. This work will help improve our ability to apply cover crops in field settings and add to important agroecological literature.

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Figures and Tables

Figure 1

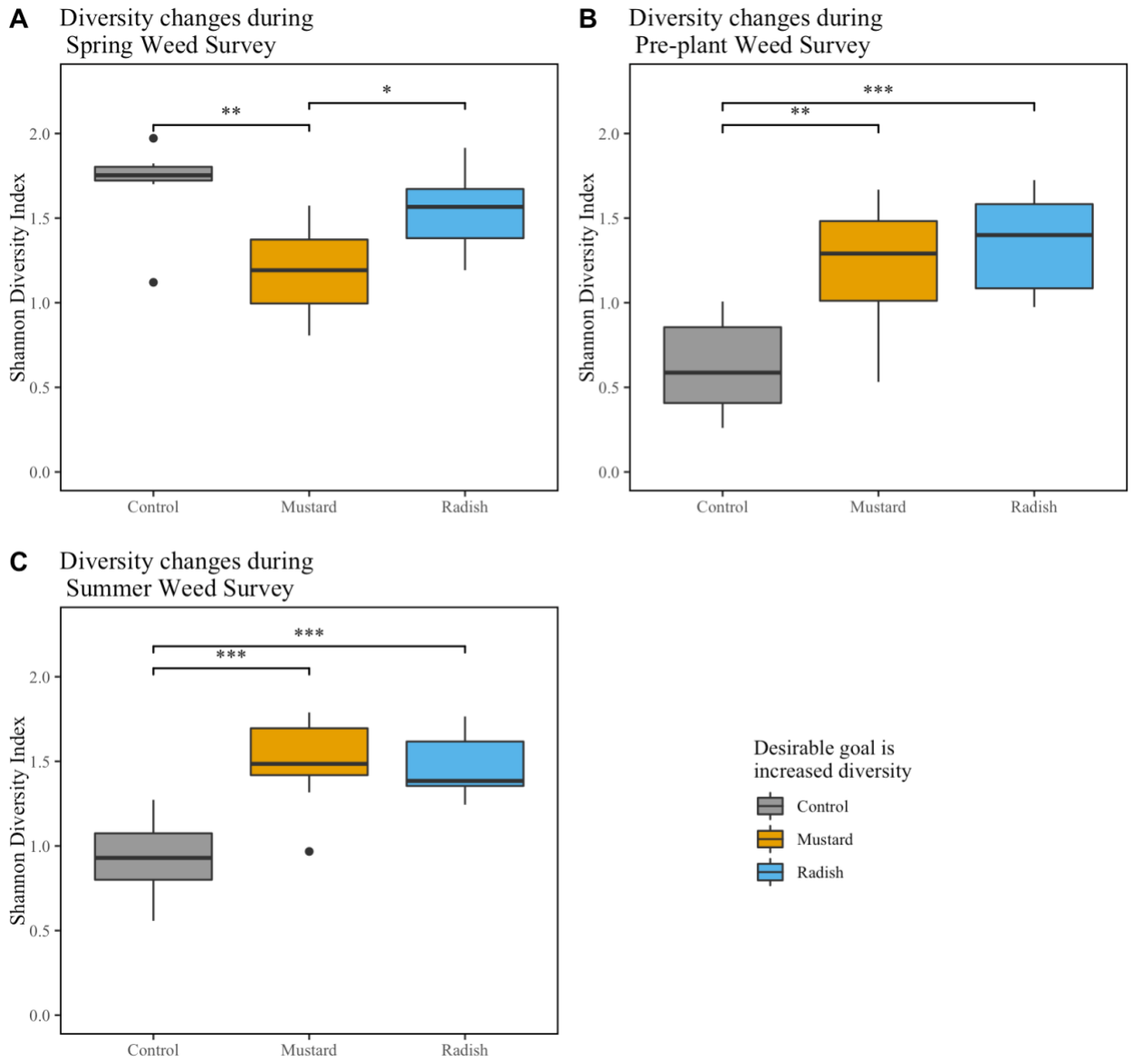

Figure 1: Weed species Shannon Diversity was reduced by mustard but not radish compared to the control in the spring weed survey ($p < 0.01$). Both mustard and radish increased weed Shannon Diversity in the pre-plant and summer weed surveys ($p < 0.01$, $p < 0.001$). The initial difference in the spring weed survey diversity is likely due to the overall high diversity of spring weeds in all treatments. Higher diversity in the later survey cover crop treatments is consistent with the domination of pigweed and lambsquarter in the control. This higher diversity is consistent with the resource pool hypothesis.

Table 1

Percent change due to cover crops differs depending on the weed species												
	Cover crops increased spring weeds in the Spring Weed				Cover crops decreased weeds in the Post Plant Weed				Cover crops decreased weeds in the Summer Weed Survey			
Weed	**mustard**	**radish**	**cover**	**M R**	**mustard**	**radish**	**cover**	**M R**	**mustard**	**radish**	**cover**	**M R**
Lamb's Quarter	-70	82	6	-84	-100*	-99*	-99*	-80	-99*	-97*	-98*	-57
Pigweed	-98*	-58*	-78*	-95	-99*	-95*	-97*	-73	-95*	-92*	-94*	-39
Grass	-17	26	4	-34	-2.7	-29	-16	38	44	28	36	12
Purslane	na	na	na	na	-98*	-87*	-92*	-86	-86*	-84*	-85*	-17
Ragweed	-39	-19	-29	-25	-70	-62	-66	-21	-37	-61	-49	62
Too small to identify	-21	118	48	-63*	26	-30	-2	80	320*	230*	275*	27
White Clover	-50	-12	-31	-44	-100	-100	-100	na	na	na	na	na
Prostrate Knotweed	146	180	163	-11	-100	-100	-100	na	na	na	na	na
Chickweed	3866*	3500*	3683*	10	na	na	na	na	na	na	na	na
Shepherd's Purse	0	525*	262	-84*	na	na	na	na	na	na	na	na
Chamomile Mayweed	inf	Inf	Inf	-100	na	na	na	na	na	na	na	na
Wild Buckwheat	-50	100	25	-75	na	na	na	na	na	na	na	na
Henbit	1710*	1485*	1597*	14.2	475	425	450*	9.5	900*	300	600*	150
Tomato	na	na	na	na	na	na	na	na	-100	-100	-100	na

Table 1: Cover crops decreased the percent abundance of most weeds, except spring weeds. Significant percent changes are marked with an asterisk, "na" denotes none of the weed species were present in the corresponding treatment, "Inf" denotes none of the weed species were present in the control. Values were calculated as percent change from the control, except M R which is the percent change radish creates from mustard.

Figure 2

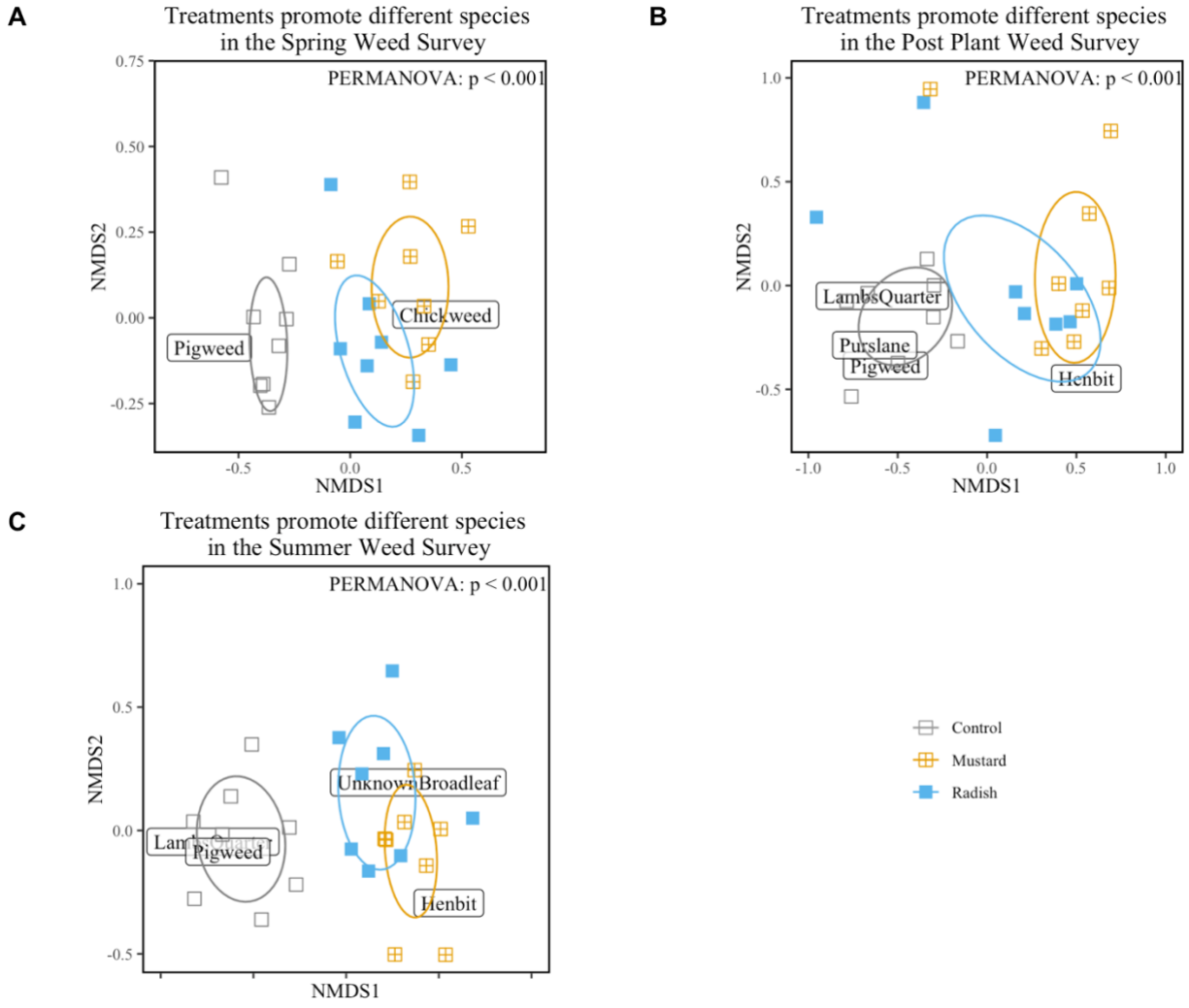

Figure 2: NMDS displays significant differences in weed community composition between control and the cover crop treatments. Indicator species for the treatments are displayed according to the NMDS identified vectors.

Figure 3

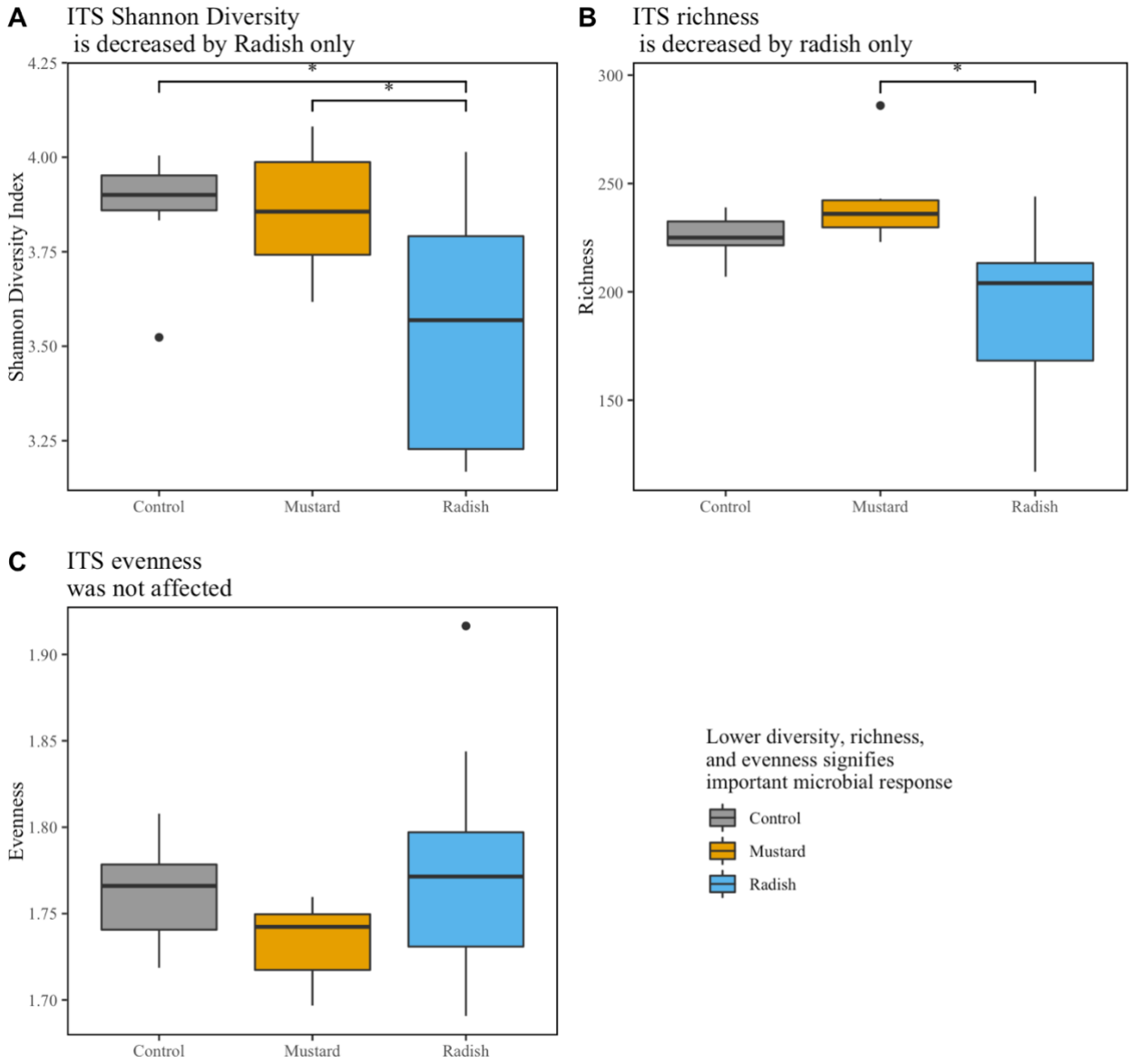

Figure 3: Fungal Shannon Diversity decreased in the cover crop treatments, but richness was only lower in the radish and evenness was not affected. Since soil samples were collected at the same time as the spring weed survey, the decreased Shannon Diversity correlates with the decreased weed diversity at that time. This correlation is consistent with the resource pool hypothesis and ecological studies that have found interactions between plant and microbial diversity.

Figure 4

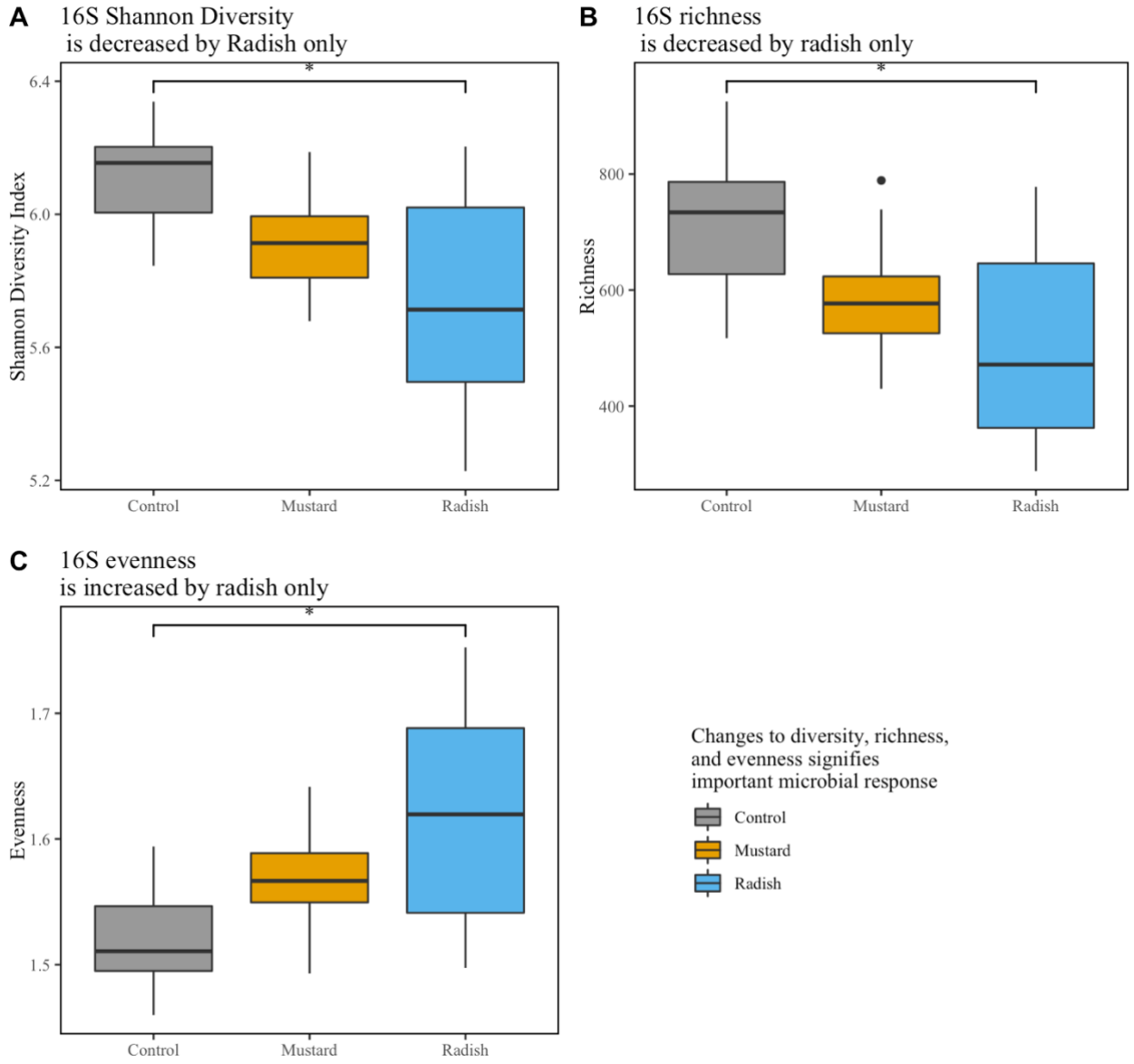

Figure 4: Bacterial Shannon Diversity, and richness were decreased, and evenness increased in only the radish treatment. Decreased diversity measurements align with the resource pool hypothesis and ecological theories that suggest plant and microbial diversity are correlated. It is unclear why the radish treatment would have a stronger effect on the bacterial diversity than the mustard cover crop.

Figure 5

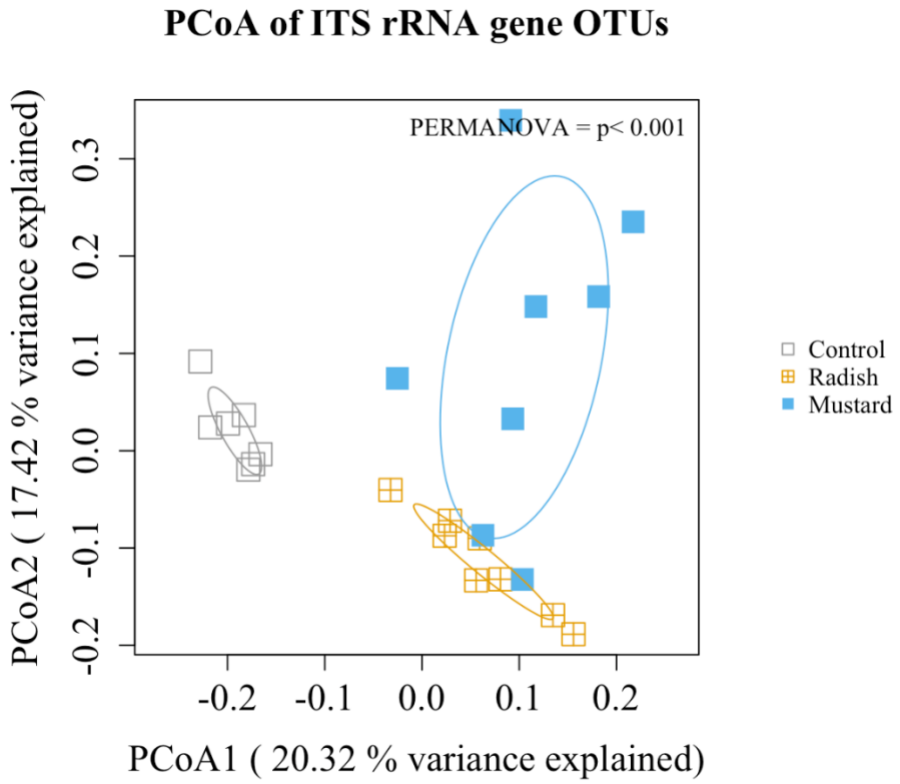

Figure 5: PCoA demonstrates differences in fungal community composition clustered based on cover crop treatments. The x and y axes represent vectors that explain 20.32% and 17.42% of the variance in the fungal dataset.

Figure 6

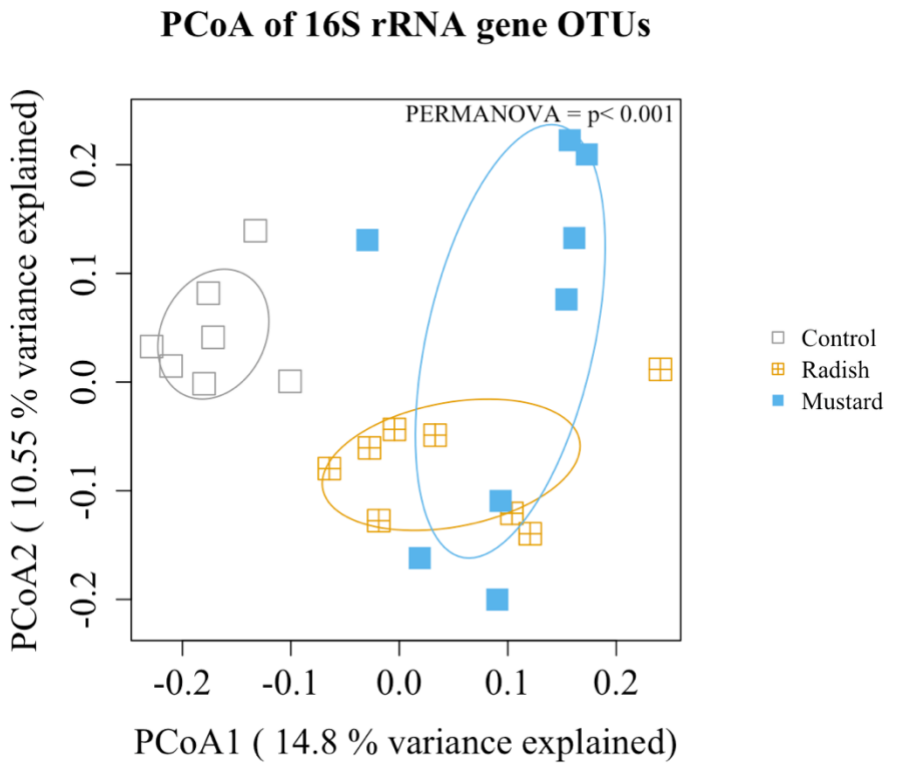

Figure 6: PCoA demonstrates differences in bacterial community composition clustered based on cover crop treatments. The x and y axes represent vectors that explain 14.8% and 10.55% of the variance in the bacterial dataset.

Figure 7

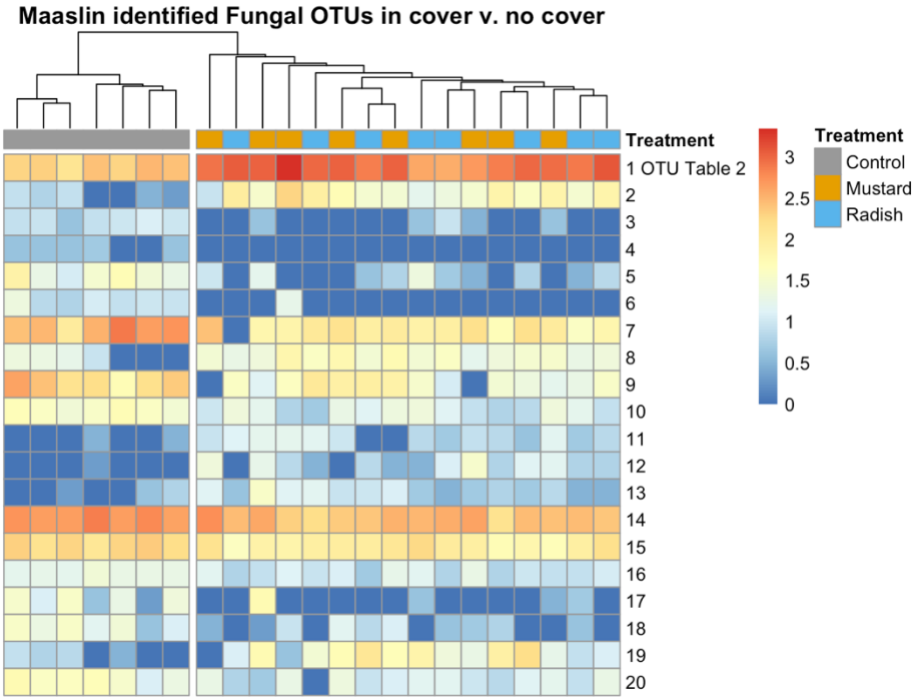

Figure 7: Maaslin2 identified fungal OTUs that are most significantly different between control and cover crop treatments. Heatmap clustering confirms the difference with two distinct groups aligned exactly with the control and mustard/radish groups. Relative abundance, displayed in blue to red tones shows which fungal OTUs were suppressed or promoted in the cover crop treatment. Taxonomic information is listed in **Table 2**.

Figure 8

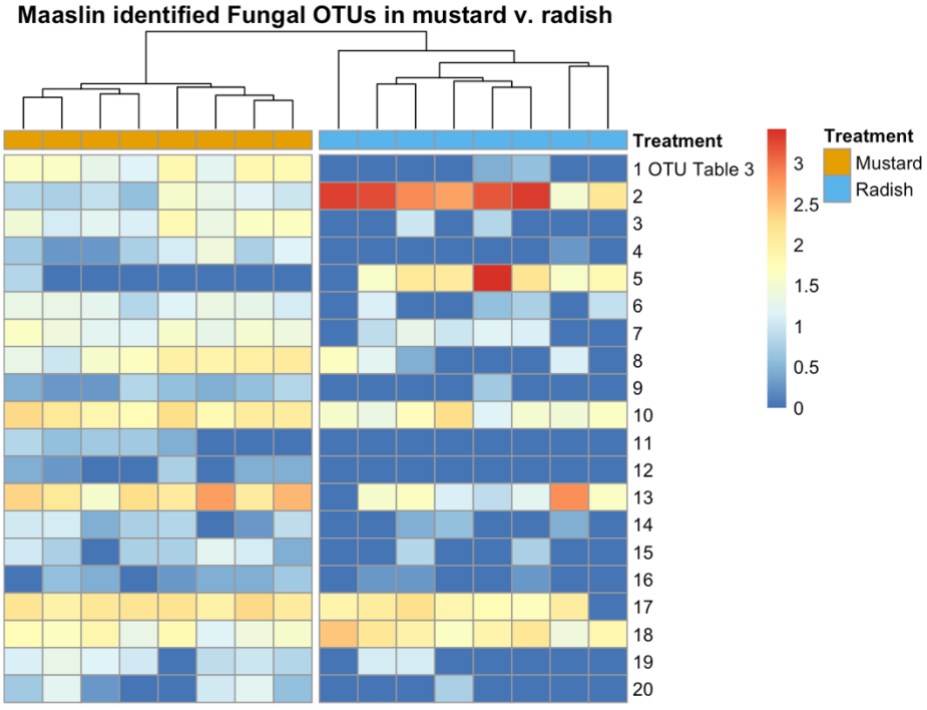

Figure 8: Maaslin2 identified fungal OTUs that are most significantly different between radish and mustard treatments. Heatmap clustering confirms the difference with two distinct groups aligned exactly with the mustard and radish groups. Relative abundance, displayed in blue to red tones shows which fungal OTUs were suppressed or promoted in the cover crop treatment. Taxonomic information is listed in **Table 3**.

Figure 9

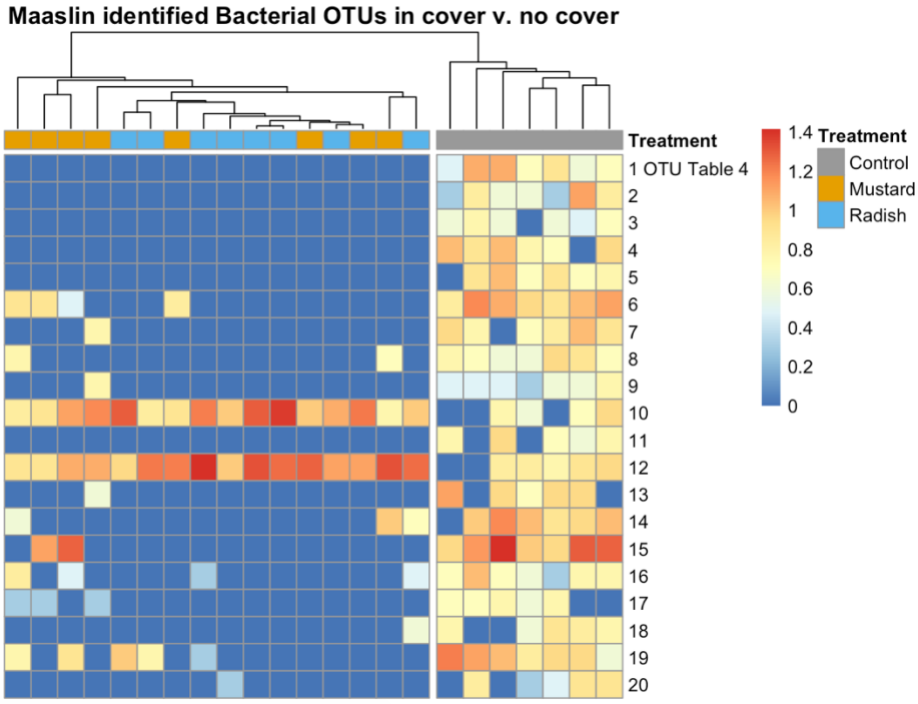

Figure 9: Maaslin2 identified bacterial OTUs that are most significantly different between control and cover crop treatments. Heatmap clustering confirms the difference with two distinct groups aligned exactly with the control and mustard/radish groups. Relative abundance, displayed in blue to red tones shows which bacterial OTUs were suppressed or promoted in the cover crop treatment. Taxonomic information is listed in **Table 4**.

Figure 10

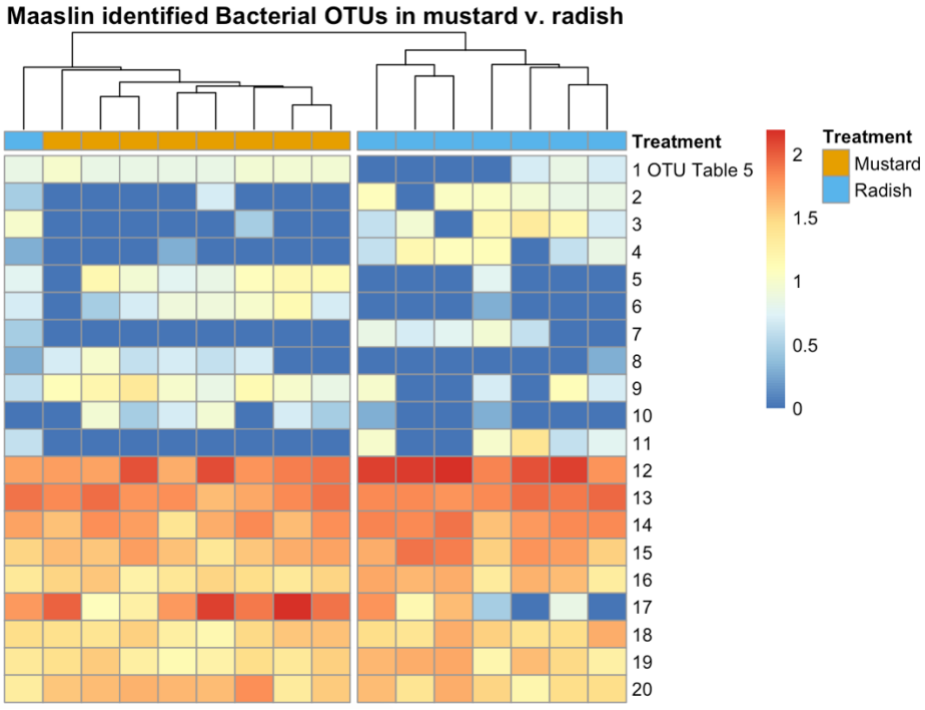

Figure 10: Maaslin2 identified bacterial OTUs that are most significantly different between radish and mustard treatments. Heatmap clustering confirms the difference with two distinct groups aligned almost exactly with the mustard and radish groups. Relative abundance, displayed in blue to red tones shows which bacterial OTUs were suppressed or promoted in the cover crop treatment. Taxonomic information is listed in **Table 5**

Supplemental Figures

Figure 11

Figure 11: Mustard cover crop residue in spring before cultivation. There is significant ground cover, with some patchy areas filled with weeds.

Figure 12

Figure 12: Radish cover crop residue in spring before cultivation. There is much less ground cover compared to mustard, with significant radish decay, leaving large holes in the soil.

Figure 13

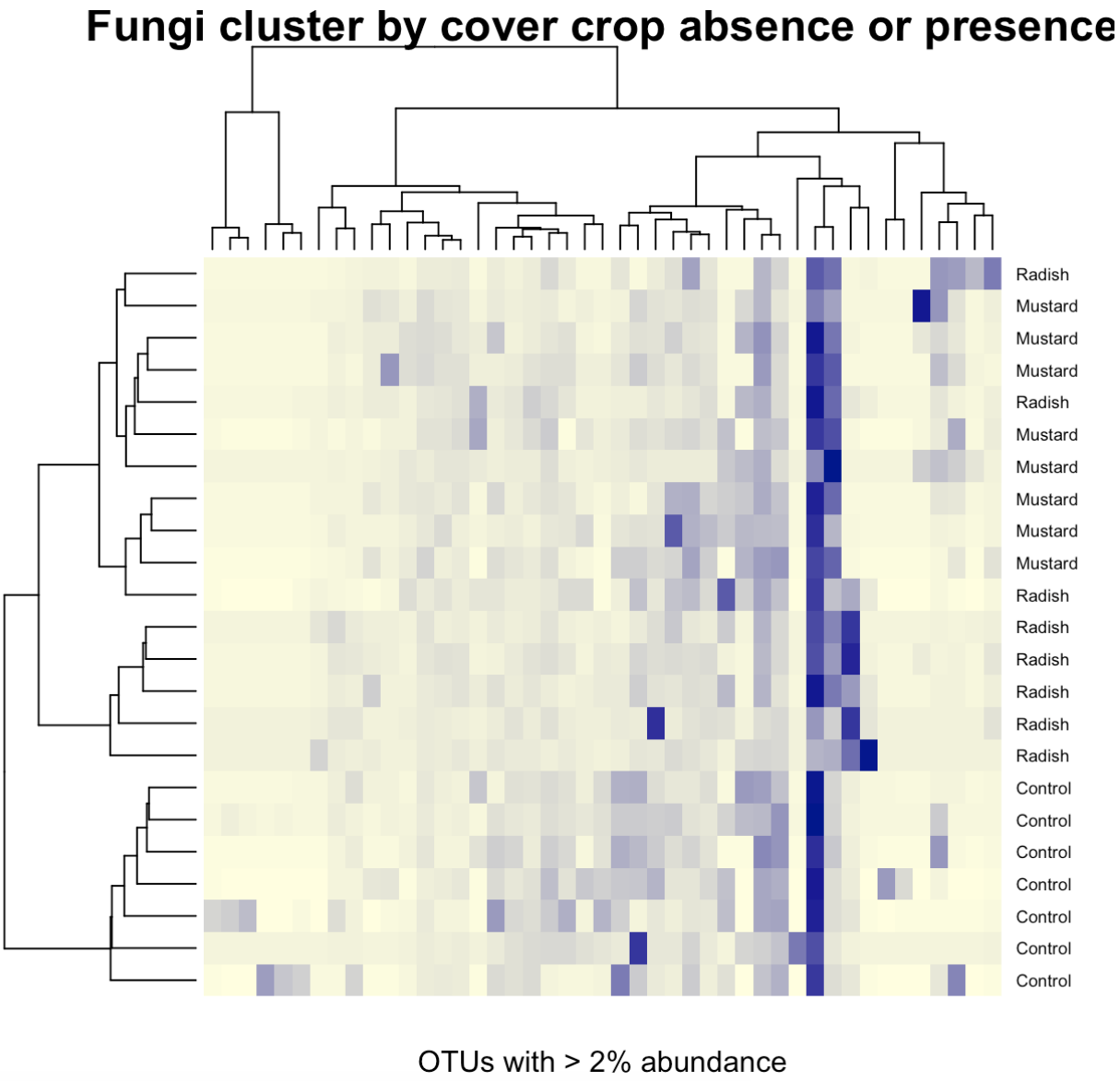

Figure 13: Based on Wards method of clustering, fungal OTUs greater than 2% abundance cluster by cover crop absence or presence. The left-hand dendrogram splits into two groups first: cover and control; then the cover splits into another two groups with mustard and radish falling mostly into one or the other.

Figure 14

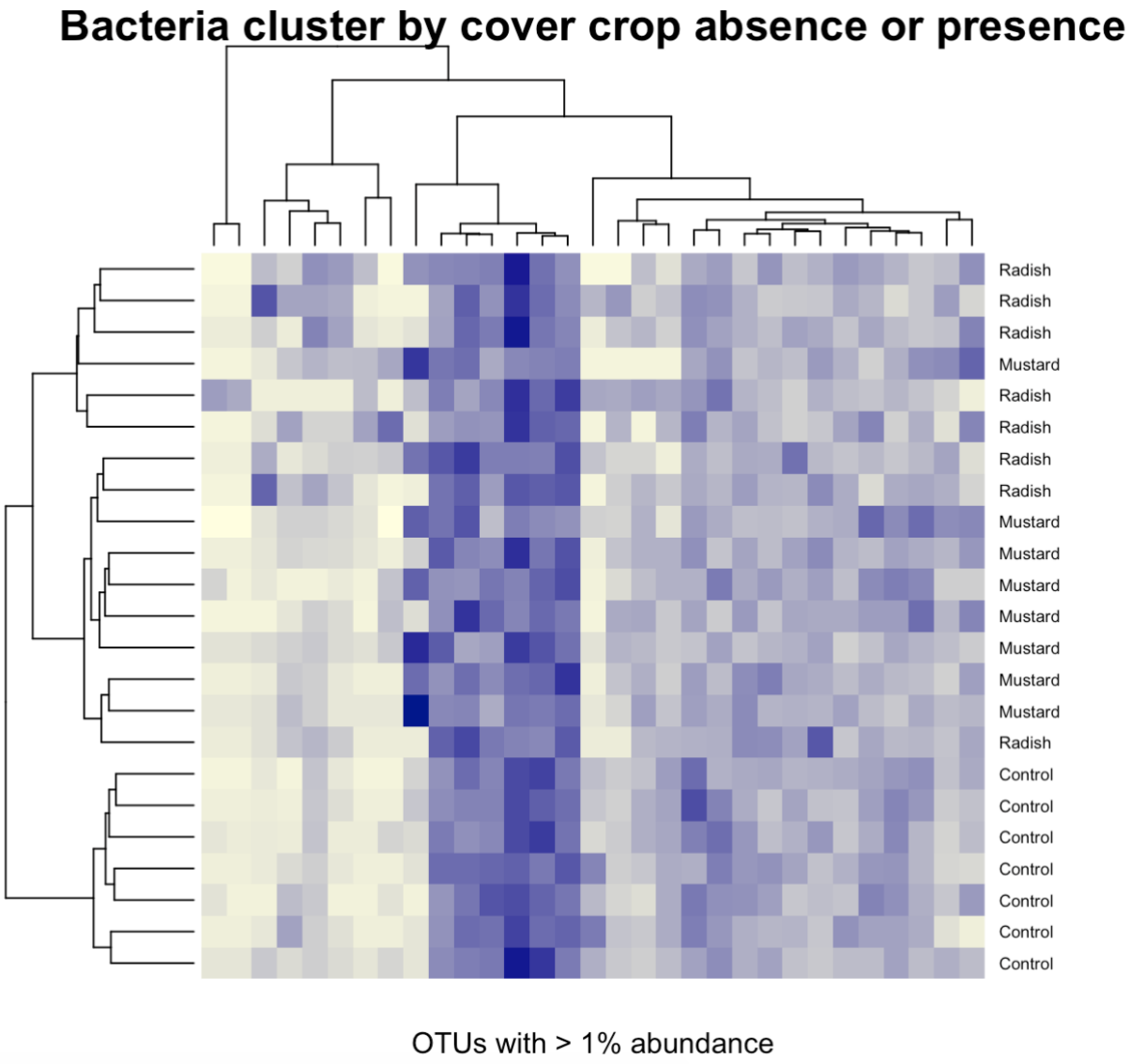

Figure 14: Based on Wards method of clustering, bacterial OTUs greater than 1% abundance cluster by cover crop absence or presence. The left-hand dendrogram splits into two groups first: cover and control; then the cover splits into another two groups with mustard and radish falling mostly into one or the other.

Table 2

Top 20 Fungal OTUs that are different between Cover and No Cover						
#	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**
1	Ascomycota	Dothideomycetes	Pleosporales	Cucurbitariaceae	Pyrenochaetopsis	Pyrenochaetopsis tabarestanensis
2	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Gibberella	Gibberella intricans
3	Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	Mortierella	
4	Ascomycota	Sordariomycetes	Hypocreales	Hypocreales	Acremonium	Acremonium persicinum
5	Basidiomycota	Tremellomycetes	Cystofilo-basidiales	Mrakiaceae	Tausonia	Tausonia pullulans
6	Basidiomycota	Tremellomycetes	Tremellales	Rhynchogas-trema taceae	Papiliotrema	Papiliotrema laurentii
7	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Aspergillus	Aspergillus clavatus
8	Ascomycota	Sordariomycetes	Hypocreales	Stachybotryaceae		
9	Ascomycota	Sordariomycetes	Sordariales	Sordariales	Staphylotrichum	Staphylotrichum coccosporum
10	Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	Mortierella	Mortierella alpina
11	Zoopagomycota	Zoopagomycetes	Zoopagales	Piptocephalidaceae	Syncephalis	
12	Basidiomycota	Agaricomycetes	Polyporales	Ganodermataceae	Ganoderma	
13	Ascomycota					
14						
15	Ascomycota	Sordariomycetes	Sordariales	Lasiosphaeriaceae	Schizothecium	
16	Basidiomycota	Agaricomycetes	Boletales	Melanogastraceae	Melanogaster	
17	Basidiomycota	Cystobasidio-mycetes	Cystobasidiales	Cystobasidiaceae	Occultifur	Occultifur mephitis
18						
19						
20						

Table 2: The top 20 most significantly different OTUs between cover crop and control, identified by Maaslin2. Maaslin2 evaluates OTUs that have statistically different relative abundances in the cover or no cover treatments. The suppression or promotion of these taxon can be found in **Figure 7.**

Table 3

Top 20 Fungal OTUs that are different between Radish and Mustard						
#	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**
1	Basidiomycota	Tremellomycetes	Cystofilobasidiales	Mrakiaceae	Mrakia	Mrakia blollopis
2	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Alternaria	
3	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales	Candida	Candida sake
4	Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	Mortierella	Mortierella elongata
5	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Neonectria	Neonectria lugdunensis
6	Ascomycota	Dothideomycetes	Pleosporales	Sporormiaceae	Preussia	unidentified
7						
8	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	
9						
10	Ascomycota	Sordariomycetes	Sordariales	Lasiosphaeriaceae	Schizothecium	
11						
12	Ascomycota	Sordariomycetes				
13	Chytridiomycota	unidentified	unidentified	unidentified	unidentified	unidentified
14	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	
15	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	Botryotrichum	Botryotrichum atrogriseum
16						
17						
18						
19	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
20	Ascomycota	Leotiomycetes	Helotiales	Leotiaceae	Neobulgaria	unidentified

Table 3: The top 20 most significantly different OTUs between mustard and radish treatments, identified by Maaslin2. Maaslin2 evaluates OTUs that have statistically different relative abundances in the cover or no cover treatments. The suppression or promotion of these taxon can be found in **Figure 8.**

Table 4

Top 20 Bacterial OTUs that are different between Cover and No Cover					
#	**Phylum**	**Class**	**Order**	**Family**	**Genus**
1	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	
2	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Skermanella
3	Actinobacteria	Acidimicrobiia	Acidimicrobiales	koll13	
4	Acidobacteria	Acidobacteria-6	iii1-15		
5	Acidobacteria	Sva0725	Sva0725		
6	Verrucomicrobia	Spartobacteria	Chthoniobacterales	Chthoniobacteraceae	
7	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	
8	Acidobacteria	[Chloracidobacteria]	RB41		
9	Acidobacteria	Acidobacteria-6	iii1-15	RB40	
10	Actinobacteria	Thermoleophilia	Solirubrobacterales		
11	Gemmatimonadetes	Gemmatimonadetes			
12	Planctomycetes	Phycisphaerae	WD2101		
13	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	
14	Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae	
15	Acidobacteria	Acidobacteria-6	iii1-15		
16	Gemmatimonadetes	Gemm-1			
17	Chloroflexi	Thermomicrobia	JG30-KF-CM45		
18	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	
19	Proteobacteria	Betaproteobacteria	MND1		
20	Chloroflexi	Chloroflexi	Roseiflexales		

Table 4: The top 20 most significantly different OTUs between cover crop treatments and control, identified by Maaslin2. Maaslin2 evaluates OTUs that have statistically different relative abundances in the cover or no cover treatments. The suppression or promotion of these taxon can be found in **Figure 9.**

Table 5

Top 20 Bacterial OTUs that are different between Mustard and Radish					
#	**Phylum**	**Class**	**Order**	**Family**	**Genus**
1	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium
2	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Cytophaga
3	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus
4	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	Nitrospira
5	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	
6	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Amaricoccus
7	WS3	PRR-12	Sediment-1		
8	Actinobacteria	MB-A2-108	0319-7L14		
9	Gemmatimonadetes	Gemm-1			
10	Cyanobacteria	Nostocophycideae	Nostocales	Nostocaceae	Nostoc
11	Cyanobacteria	Oscillatoriophycideae	Oscillatoriales	Phormidiaceae	Phormidium
12	WS3	PRR-12	Sediment-1		
13	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
14	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	
15	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Thermomonas
16	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	
17	Actinobacteria	Actinobacteria	Micrococcales		
18	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Skermanella
19	Actinobacteria	Acidimicrobiia	Acidimicrobiales	EB1017	
20	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	Nitrospira

Table 5: The top 20 most significantly different OTUs between mustard and radish treatments, identified by Maaslin2. Maaslin2 evaluates OTUs that have statistically different relative abundances in the cover or no cover treatments. The suppression or promotion of these taxon can be found in **Figure 10.**

CHAPTER TWO

Glucosinolate mustard seed meal reduce plant persistence across functional types and alter soil microbial composition and activity

Abstract

Glucosinolates are volatile compounds released by mustard cover crops that can alter the soil biological and chemical properties through both metabolite and carbon influx into the soil, leading to dramatic changes in microbial activity and species composition. In this greenhouse project, we used Brassica hirta, cv. 'Tilney' (yellow mustard) seed meal as a source of glucosinolates to target glucosinolate-mediated responses on plant growth. We tested whether glucosinolates suppress germination and growth differently across plant functional groups, revealing potential impacts on plant communities. We also hypothesized that glucosinolates alter soil microbial community composition and activity, which play important roles in the critical period of seed germination and persistence. We examined three plant families, Fabaceae, Amaranthaceae, and Brassicaceae to assess differential responses to glucosinolates based on functional characteristics of nitrogen-fixing, nitrogen-responsive, and non-mycorrhizal plants. Mustard seed meal (MSM) suppressed weed presence in all three plant families with an average of 0.15 individuals in the MSM treatment, and 0.78 in the non-MSM control. Additionally, all three families were suppressed by MSM, ranging from 69% to 95% reductions which indicate the non-selective control of glucosinolates. Soil microbial activity, as measured by CO_2 respiration, remained high under MSM for 7 days and declined precipitously to indicate microbial utilization of MSM in soils. Soil microbial diversity was reduced by 13% with distinctly different compositions under MSM additions. The results indicate that glucosinolates are effective in suppressing plant growth and altering microbial composition, but the effects were non-selective with similar responses across species and taxonomic groups.

Introduction

Mustard cover crops could lead to agriculturally and ecologically significant weed control, pathogen suppression and soil microbiome alterations through both glucosinolate and carbon influx into soil (Berkowitz et al., 2022). Volatile compounds called glucosinolates from

mustard biomass, seeds and root exudates break down into biocidal isothiocyanates (*p*-hydroxybenzyl isothiocyanate in Tilney) (Gamba et al., 2021; McCully et al., 2008; Tsunoda et al., 2018). Cover crop biomass and root exudates also stimulate microbial activity and create more diverse resource pools (Smith et al., 2010), resulting in more diverse weed and microbial communities (Carney and Matson, 2006; MacLaren et al., 2020; Storkey and Neve, 2018; Venter et al., 2016; Vukicevich et al., 2016). One challenge to adopting brassica cover crops for these applications is that we do not know the extent to which microorganism responses to glucosinolate or carbon drive weed reduction.

Glucosinolate research has resulted in a range of applications for weed and soil pathogen control. Evidence shows the compounds can effectively control weeds including Redroot Pigweed, Shepherd's Purse, Purslane, Common Barnyard grass, Rembrandt Tall Fescue, Evening Shade Perennial Rye, Riviera bermudagrass, Wild Oat, Italian Ryegrass, Prickly Lettuce, Kochia, and Green Foxtail (Berkowitz et al., 2022; Brown and Morra, 1996; Earlywine et al., 2010; Handiseni et al., 2011; Krishnan et al., 1998; Pużyńska et al., 2019). Glucosinolate has also been applied to effectively control soil-borne pathogens damaging to cucumbers (*Fusarium oxysporum*), apples (*Penicillium expansum* and *Botrytis cinerea*), wheat (*Gaeumannomyces graminis var. tritici, Rhizoctonia solani, Fusarium graminearum* and *Bipolaris sorokiniana*), and strawberries (*Macrophomina. phaseolina*), to name a few (Hollister et al., 2013; Jin et al., 2019; Kirkegaard et al., 1996; Kirkegaard and Sarwar, 1998; Mazzola et al., 2017; Wu et al., 2011). The soil microbiome as whole can also be impacted by glucosinolates with reduced abundance, diversity, and activity (Hollister et al., 2013). These effects can be short-lived, and are often gone or significantly reduced within the scale of weeks or months (Hannula et al., 2021; Hollister et al., 2013; Zaccardelli et al., 2013). Most of these studies focus on the varying weed and soil microorganism responses that occur due to different Brassica species with ranges of glucosinolate types and concentrations.

Though some of these studies utilize purified glucosinolate or isothiocyanate compounds, studies that use cover crop plants, biomass, or seed meal have compounding factors, such as carbon, that may also play a role in the weed and microorganism response. Increased carbon from cover crop biomass or seed meal have been found to decrease *Acidobacteria* and increase *Bacteroidetes* and *ß-Proteobacteria* (Fierer et al., 2007); increase

microorganism abundance (27%), activity (22%), and diversity (2.5%) according to a recent meta-analysis (Kim et al., 2020); and increase nitrogen immobilization through stimulating microorganism activity (Gannett et al., 2022, Mooshammer et al., 2014).

Despite the fact that Brassica plants are one of the most researched family of plants that utilize chemical defense mechanisms (Plaszkó et al., 2021), few studies have sought to connect microorganism and weed responses. There is some evidence to suggest that soil microorganisms play an important role in the allelopathic interactions between glucosinolates and weed suppression (Hoagland et al., 2008; Rehman et al., 2013). Hoagland et al. found glucosinolate driven increases in *Pythium spp.* were correlated with increased weed seedling mortality (Hoagland et al., 2008). Studying both the microbial community and weed responses together could identify different dynamics caused by the glucosinolate and carbon compounded mechanisms behind Brassica cover crop weed control.

Glucosinolate induced microbial changes may selectively cause negative plant-soil feedback to weeds from different families while causing positive plant-soil feedback to other Brassica weeds. Plant-soil feedbacks (PSFs) occur when one plant influences the soil microbiome, resulting in subsequent plant growth to be altered (Miller et al., 2019). PSF research is most established in grassland and forest environments and demonstrates the phenomena that a plant species or family conditions the soil to simultaneously decrease heterospecific weed competitiveness and increase conspecific weed fitness. (Hannula et al., 2021; Van der Putten et al., 2013). Agricultural application of PSF ecological research, including those focusing on Brassica families supports this theory in certain circumstances (Lankau et al., 2011; Lankau and Strauss, 2007; Oduor et al., 2020, 2017). Lankau and Strauss found that as the community became more heterospecific, the selective value of sinigrin (volatile compound released by *B. nigra*) increased (Lankau and Strauss, 2007). They hypothesized that this response may be because Brassica plants do not form relationships with arbuscular mycorrhizal fungi while most other species do. If glucosinolate is the driving mechanism, we predict the weed response will follow the PSF theory and select against heterospecific weeds, but not affect other Brassica weed species.

On the other hand, carbon induced microbial changes may select against nitrophilous weeds. Carbon influx provides a food source for microorganisms, causing the microbial activity

to dramatically spike (Chen et al., 2003; Kallenbach et al., 2019). With this increase metabolization, microorganisms immobilize nitrogen in their bodies (Chen et al., 2003; Mooshammer et al., 2014) causing selective pressure against nitrophilous weeds like amaranths (Gannett et al., 2022). On the other hand, nitrogen fixating organisms and the associate nitrogen fixing weeds like legumes may be stimulated because they are able to take advantage of the carbon in a nitrogen limited environment. If carbon addition is a driving mechanism, we expect to see reduced nitrophilous weeds and increased nitrogen fixing weeds due to their demands for inorganic nitrogen to support rapid growth.

Weeds responded to brassica cover crops were consistent with both the carbon and glucosinolate mechanisms in the field experiment of Chapter 1. Significant nitrophilous weed reduction including Pigweed, Lambsquarter, and Purslane; combined with different bacterial and fungal community composition warrants further investigation. To better control variation inherent in field experiments, we conducted a greenhouse experiment that investigates the dynamics between the soil microbiome and weed species control. The experiment assessed the effects of mustard seed meal on three different weed families: Amaranthaceae, Brassicaceae, Fabaceae, and the bacterial community. We tested whether MSM reduces weed growth, and if some weed families are more affected than others depending on the dominant microbial mechanism. Based off the findings in Chapter 1 and the PSF ecological theory, if glucosinolate is the main mechanism, we hypothesized Brassicaceae weeds would be least effected by the MSM treatment with a decrease in bacterial diversity, microbial respiration, and plant pathogens. If carbon is the driving mechanism, we hypothesized the Amaranthaceae weed family would be promoted, while the Legume weed family would be most reduced by MSM. Carbon would also increase bacterial diversity, microbial respiration, carbon loving bacteria, and nitrogen fixing bacteria.

Materials and Methods

The experiment was conducted in a greenhouse at Cornell University, Ithaca NY. Weed species were planted in pots and grown under limited nutrient conditions for 42 days. We observed weed germination and soil CO_2 respiration throughout the experiment. Weed biomass and soil samples for bacterial community composition were collected at the end of the experiment.

A randomized complete block design (RCBD) was used with two factors: glucosinolate (present or not), and weed family (Amaranthaceae, Brassicaceae, Fabaceae). The glucosinolate treatment was achieved using MSM at double the amount generally observed in mustard biomass. The three weed families were chosen for their functional purposes: Amaranthaceae as a nitrophilic group, Brassicaceae as a non-arbuscular mycorrhizae fungi (AMF) relationship forming functional group, and Fabaceae as a N fixing group. The weed families contain three different weed species each, chosen for their agricultural importance and based on patterns observed in Chapter 1 (Berkowitz, et al., 2022).

The experimental design consisted of eight replicate blocks for each of the six treatment combinations. Each replicate block contains all nine weed species. The Amaranthaceae weed species were *Amaranthus retroflexus* (Redroot Pigweed), *Chenopodium album* (Lambsquarters), and *Amaranthus palmeri* (Palmer Amaranth). The Brassicaceae weed species were *Barbarea vulgaris* (Yellow Rocket), *Capsella bursa-pastoris* (Shepherds Purse), and *Sinapis arvenis* (Wild Mustard). The Fabaceae weed species were *Trifolium repens* (White Clover), *Trifolium pratense* (Red Clover), and *Medicago lupilina* (Black Medic). Before planting, soil was amended with MSM at 200 g/m^2 of soil (Minn-dat growers, Grand Forks, ND). This equates to, at most, approximately 6% of the methyl-isothiocyanate found in the fumigant Vapam (Amvac Chemical Corporation, Los Angeles, CA) (Morra and Kirkegaard, 2002). Glucosinolates break down into methyl-isothiocyanates when they come in contact with water. The rate of the MSM was chosen so that we would observe weed responses, remain consistent with cover crop levels, and stay lower than the fumigant methyl-isothiocyanate concentration (Björkman et al., 2015; Norsworthy and Meehan, 2005). A field relevant soil microbiome was introduced into the soil using a field soil slurry as used in (Panke-Buisse et al., 2015). Soils were amended with AMF as instructed by the manufacturer (Premium Mycorrhizal Inoculant, DYNOMYCO, Israel) and alfalfa and clover rhizobia inoculant at 8.7 x10^{-4} oz/g soil (N-Dure Premium, Walmart). The MSM, field soil slurry, AMF inoculant, and rhizobia inoculant were applied at the same time, and watered, triggering the biocidal breakdown of glucosinolate.

An experimental unit is one 4” pot. In each pot, we planted six seeds of the appropriate weed. Pots were fertilized at 20 ppm N twice a week and watered as needed. Due to a

greenhouse staff error, the pots received 300 ppm N one time right as weeds were germinated. The mistake was adjusted by watering with clear water the rest of the week and only fertilizing with 20 ppm N once the following week. Weed seed germination was tracked every other day beginning 5 days after planting. Weeds were thinned to one plant per pot after each germination measurement. At harvest weed shoots were separated from the roots and dried in an oven set to 80°C then weighed. We calculated the percent change of weeds due to MSM [(MSM-control/control)*100]. Weed plant presence was analyzed using a logistic model predicting probability of plant presence from the interaction of weed family and MSM treatment (function *glm* with a biological family and logit link, R version 4.6-4.14). Weed biomass was analyzed using ANOVA and Tukey-HSD post-hoc analyses.

Soil subsamples were stored in jars at the beginning of the experiment CO_2 respiration was measured without plants at days 2, 5, 7, 14, and harvest. At the harvest timepoint, both soil respiration from the subsamples (without plant) and the greenhouse (with plant, after harvesting) were measured. For all measurements, soil was placed in mason jars with rubber stoppers fitted in their cap. If needed, soil was hydrated with equal amounts of water, shaken, and allowed to normalize. Accumulated CO_2 was removed from the jars to achieve a zero point, jars were allowed to rest for one hour, then respiration measurements were taken using a CA-10a carbon dioxide analyzer (Sable Systems Intl., Las Vegas, NV). While resting, a standard curve was created with 1mL, 0.8 mL, 0.6 mL, 0.4 mL of both 0.453% and 5.001% CO_2.

To analyze results, a linear equation was created using the standard curves measurements, with the mL CO_2 as the independent variable and the measured peak areas as the dependent variable. We then used this equation to calculate the mL CO_2 from the peak area measurements. We adjusted the mL CO_2 based on soil weight and volume, atmospheric CO_2, jar volume and incubation time ([(sample mL CO_2 – atmospheric mL CO_2)*(jar volume mL – soil volume mL))/soil mass g] to report mL CO_2 per g soil produced in one hour. CO_2 fluctuation was analyzed using t-tests, ANOVAs and Tukey HSD post-hoc tests when appropriate.

At harvest, rhizosphere soil samples for bacterial community composition were collected and stored in a refrigerator set to 4°C until processed. Six samples from the eight replicate blocks for each of the six treatment combinations were analyzed for bacterial

community composition. Using the manufacturer instructions from the Qiagen DNeasy extraction kit (Beverly, MA), DNA extractions were conducted from approximately 2.5 g of soil. The 16s rRNA V3-V4 region of bacteria was assessed using high-throughput sequencing and then amplified by running Polymerase Chain Reactions (PCR) on a Bio-Rad C1000 Thermal Cycler (Hercules, CA). Reactions used 5 µL of DNA, 10 µL of Quantabio Accustart II PCR SuperMix (Beverly, MA), 0.5 µL of both forward and reverse primers for the 16S region, and 4 µL nuclease-free water for a total volume of 20 µL. Amplicons were cleaned, sequenced, prepared, and clustered as stated in Chapter 1 (Berkowitz, et al., 2022).

All further microbial analyses were conducted in R version 4.1.2 (R core team, 2022) following the same protocol as Chapter 1. Bacterial and fungal samples were rarified (randomly sample a set number of reads) to match the minimum reads of operational taxonomic units (OTUs) present (rarify function, package vegan, version 2.5-7). Bacterial and fungal community richness, evenness and Shannon Diversity were calculated on OTU percent abundance [(OTU/Total OTUs)*100], and analyzed for treatment effects using ANOVA and Tukey post-hoc test. A PERMANOVA using 999 permutations was run on the Bray-Curtis distances to quantify statistical differences between treatments. Then, Principal Coordinates Analysis (PCoA) was conducted to assess community differences. Cluster analysis using Wards method was used to validate PCoA groupings (Landau and Chis Ster, 2010). We used the machine learning algorithm randomForest (fuction *randomForest*, in package randomForest, version 4.6-14) to predict the treatment based off OTU percent abundance with or without rare genera removed. This algorithm then displays a subset of OTUs that are most predictive for each treatment. The function Microbiome Multivariable Association with Linear Models 2.0 (Maaslin2) was also used to identify OTUs associated with each treatment and validate the randomForest results. Maaslin2 conducts linear and mixed models to compare the percent abundance of each OTU between treatments. It then identifies OTUs that have significant differences, either present or not present, in each treatment and adjusts for multiple hypothesis testing (Mallick et al., 2021). Rather than a predictive decision tree algorithm like randomForest, Maaslin2 begins with treatment groups and then identifies OTUs that are promoted or repressed in a treatment.

Results

Weed Suppression

Weeds germinated differently depending on MSM treatment and weed species, this germination difference could be due to both the germination rates of the species and the MSM treatment. *Sinapis arvenis* (Wild Mustard) was removed from the study due to germination failure in the control. To measure MSM treatment effects we analyzed weed growth through the absence or presence of a plant at the end of the experiment and the biomass produced. Mustard seed meal suppressed weed presence in all three weed families with an average of 15% weeds present in the MSM treatment, and 88% weeds in the control ($p < 0.001$). Amaranth species decreased by 74% ($p < 0.001$), crucifer species decreased by 69% ($p = 0.003$), and legume species were decreased by 96% ($p < 0.001$) (**Figure 1**). MSM reduced the legume family the most ($p = 0.03$), and amaranth and crucifer were affected the same ($p = 0.92$). Mustard seed meal also reduced weed dry biomass in all three weed families with an average of 0.002 g in the MSM treatment and 0.34 g in the control ($p < 0.001$). Amaranth species decreased by 100% ($p < 0.001$), crucifer species decreased by 98% ($p < 0.001$), and legume species were decreased by 100% ($p = 0.025$) (**Figure 2**). MSM induced weed biomass reductions differed with legume reduced less than amaranth and crucifer ($p < 0.001$, $p = 0.002$), amaranth and crucifer were reduced the same ($p = 0.31$). Some species growth was affected more than others, as listed in **Table 1,** and visualized in our sampling pictures in **Figure 8**.

Bacterial Activity and Community Composition

Soil respiration was significantly increased by MSM in samples without a plant in all time points ($p < 0.001$ for all, **Figure 3**). Samples with plants displayed the opposite, MSM significantly decreased soil respiration ($p < 0.001$) with an average of 3.66 mL CO_2/g/hour, and 11.38 mL CO_2/g/hour in the control (**Figure 4**). Weed family and species did not affect the soil respiration alone or interacting with MSM ($p = 0.215$ and 0.552, and $p = 0.10$ and $p = 0.88$ respectively).

To maintain high data quality, some of the samples for the bacterial dataset were removed due to various sequencing and experimental issues. In total, 61 samples were included with 1,633 unique bacterial sequences observed. MSM reduced Shannon Diversity by 13% (p

= 0.019), species richness was decreased in only the crucifer weed family (MSM p = 0.087, crucifer p < 0.001), and evenness was not affected (p = 0.817), see **Figure 5**. Weed family did not have any significant effects on Shannon Diversity, or evenness (p = 0.656, p = 0.843).

The PCoA (**Figure 6**) demonstrates bacterial community composition was significantly different due to MSM treatment, with supporting PERMANOVA (p = 0.001) and equal dispersion (0.204). The first two vectors of the PCoA explain a total of 58% of the variance in the dataset. Weed family and weed species did not alter the bacterial community composition (PERMANOVA p = 0.366, p = 0.126). Cluster analysis displayed in **Figure 9** identified two distinct clusters, the MSM samples were in one cluster along with three control samples, while the control was in another. This cluster analysis validates the PCoA microbiome treatment grouping.

The machine learning algorithm, randomForest, randomly creates decision trees based off microbial OTUs to identify OTUs most associate with MSM and control samples. These decision trees create a model based on a subset of data that can predict a treatment based on the predictor OTUs. The model is validated utilizing the entire data set, and percent error is reported. We conducted two randomForest classifier tests, one on the rarified OTU percent abundance and another with rare genera removed from the rarified OTU percent abundance. Both yielded highly accurate decision models with 0% error rate. The top 20 most predictive OTUs from the dataset including rare genera are listed in **Table 2**. To validate these predictive OTUs, Maaslin2 was used to identify OTUs that are statistically different between treatments, rather than predictive features like randomForest. Maaslin2 identified 12 of the same 20 predictive OTUs as randomForest, listed in **Table 3**. The top 20 bacterial OTUs clustered well with all samples clustering within their respective sample groups (**Figure 7**). Of the 12 common OTUs identified by both randomForest and Maaslin2, three were not able to be classified, MSM promoted OTUs from the family *Thermodesulfoyibrionaceae, Verrucomicrobiaceae,* and *Rhodobacteraceae,* and suppressed OTUs from the phyla *Proteobacteria,* and families *Verrucomicrobia*, and *Planctomycetaceae.*

Bacteria taxon responses to carbon in literature such as decreased *Acidobacteria* and increased *Bacteroidetes* and *ß-Proteobacteria* populations (Fierer et al., 2007) were assessed using ANOVA and Tukey post-hoc tests of relative abundances. Seven phyla were identified

in our dataset including: *Acidobacteria, Proteobacteria, Nitrospirae, Verrucomicrobia, Planctomycetes, TM6,* and *Euryarchaeota.* Consistent with the literature MSM reduced the relative abundance of *Acidobacteria* by 88% ($p < 0.001$) and increased the relative abundance of *Proteobacteria* by 41%. MSM also reduced *Nitrospirae* by 41% ($p < 0.001$), *Verrucomicrobia* by 71% ($p < 0.001$), and *TM6* by 87% ($p < 0.001$). *Planctomycetes* and *Euryarchaeota* were not significantly affected ($p = 0.606$, $p = 0.161$).

Discussion

Weed Growth Reduction

Weed growth was reduced by MSM as hypothesized, though the families affected does not necessarily support or rule out carbon or glucosinolate as driving mechanisms. We hypothesized if carbon is the driving mechanism, nitrophilous weeds like Amaranths would be reduced, while nitrogen fixing plants like Legumes would increase. Weed dry biomass supports this hypothesis with the Legume weed family dry biomass reduced the least by MSM (**Figure 2**). On the other hand, in weed plant presence the Legume family was reduced the most (**Figure 1**), which does not support the expected response to carbon influx. It is possible that rhizobia soil inoculation did not effectively establish before MSM application, which would prevent the Legume family from partnering with nitrogen-fixing bacteria due to issues with experimental soil inoculation. This would remove their competitive advantage that this hypothesis hinges on. Soils were inoculated with alfalfa and clover rhizobia inoculant, common genera include *Rhizobium, Mezorhizobium, Ensifer (*formerly *Sinorhizobium), Bradyrhizobium, Phyllobacterium, Microvirga, Azorhizobium, Ocrhobactrum, Methylobacterium, Devosia, Shinella (Class of α- proteobacteria), Burkholderia, Cupriavidus (*formerly *Ralstonia)* (Berrada and Fikri-Benbrahim, 2014). None of these genera were identified in the bacterial dataset, which may be due to limitations with taxonomic identification (Sentausa and Fournier, 2013), or due to the inoculant's failure to compete with the soil slurry inoculation (Gadhave et al., 2016). Even if the inoculants did establish, nodulation can occur between 14 and 100 days, so it is possible the plants were not able to form nodulation before harvest (Maj et al., 2009; TRINICK and HADOBAS, 1990). Given that control Legume plants were still able to establish, and the MSM prohibited germination before Legume plants had any chance to form nodules, it

is more likely that MSM played a major role in reducing their growth. The higher decrease in Amaranth weed biomass supports carbon as a mechanism, but dramatic reduction in all three weed families associated with MSM suggests that carbon may not be the most important mechanism.

We hypothesized that if glucosinolate is the driving mechanism, Brassicaceae family weeds would be less affected by the MSM compared to other families due to positive plant-soil feedback. We did not observe any positive PSFs, with the Brassica weed family reduced equally to Amaranth and Legume. Though we did not observe positive PSF, this alone is not enough to rule out glucosinolate as a driving mechanism. PSF research exhibits allelopathic positive effect on conspecific plants only in certain circumstances. In a review paper, Van der Puten et al. report that conspecific seedlings are still susceptible to mature trees, which may play a role in maintaining genetic diversity in forests (Van der Putten et al., 2013). It is also possible the positive PSF only occurs for plants of the same *species*, and that plants in the same *family* are still susceptible to the negative PSFs. Miller et al. reported this negative effect on different grassland species, some in the same family (Miller et al., 2019). Therefore, the weed results do not support the hypothesis that positive PSF would preserve plants within the same family, but that does not rule out glucosinolate as a driving mechanism.

Consistent with other glucosinolate studies, all weeds were reduced dramatically by the MSM treatment. This is promising for growers that are seeking natural herbicides as alternatives to chemical options, but the absence of selective effects on certain species may be a major risk for the cash crop. Further mechanistic understanding is necessary before recommending Brassica cover crops or glucosinolate applications to growers. As evidenced in this study, observing impacts on different weed species alone is not sufficient to identify the underlying mechanisms that cause this reduction. Assessing microbial community composition helps to address this limitation, but more genetic and plant anatomy research is necessary to understand the biocidal effects before widespread field application.

Microbial Activity and Bacterial Community Composition

MSM was associated with a significantly higher soil respiration without plants, but significantly lower with plants. This discrepancy could be due to the short-lived effects of carbon additions from MSM, and additional carbon provided by plants. In the samples without plants, the soil respiration dramatically decreases after seven days, suggesting that the mustard seed meal is the primary food source. After one week, the microorganisms exhaust the seed meal, and the activity decreases significantly without a food source. which drives microbial activity until the carbon source is exhausted after one week. This is consistent with other seed meal studies that observe microbial changes in only the first week after amending soils with seed meal (Hannula et al., 2021; Hollister et al., 2013; Zaccardelli et al., 2013). Plants also exude carbon into the soil, providing food sources to increase microbial activity. Since the MSM treatment so effectively reduced all weeds, it is possible that the plants in the control stimulated microbial activity, while the lack of plants in the MSM treatment resulted in the observed microbial activity reduction. Any stimulation from the initial MSM amendment in the plant samples would have spiked in the first week, before CO_2 measurements were collected.

The observed microbial activity suggests that carbon is an important mechanism driving changes to the microbial community. The initial spike in microbial activity may mean that effects of MSM on weed community or microbial community composition only occur within this short window (Hannula et al., 2021; Hollister et al., 2013). Due to the impartial nature of the MSM weed suppression, this short-term effect may be promising for growers. Application could be timed so that the suppressive effects are maximized against weeds or even pathogens but peak before the cash crop is planted.

MSM may selectively stimulate or suppress certain microorganisms in ways that help identify carbon or glucosinolate mechanisms. Clear differences between the bacterial community compositions of MSM and control (**Figure 6**) support the possibility of allelopathic connections between the weed responses and soil microbiome. Both glucosinolate and carbon could have played a role in the bacterial responses, but nuanced differences in diversity and taxonomy could help distinguish the two mechanisms. We hypothesized that if carbon was a mechanism, we would observe increased bacterial diversity, carbon loving bacteria and nitrogen fixing bacteria. On the other hand, if glucosinolate is a driving mechanism, we

hypothesized a decrease in bacterial diversity, and plant pathogens. Contrary to expected carbon results, but supportive to the expected glucosinolate results, we observed a significant decrease in both Shannon Diversity and richness due to the MSM amendment (**Figure 5**).

Carbon may still be an important mechanism, even with the reduced diversity. Though increased resource ability often leads to increased diversity, after a certain threshold, specialists uniquely fit to digest carbon are promoted (Mittelbach et al., 2001). It is possible the carbon from the MSM was past this threshold and rather than increasing bacterial diversity, specialist carbon loving bacteria or nitrogen immobilizing bacteria began to dominate. This was not the case, in fact of the 12 OTUs identified by randomForest and Maaslin2 and randomForest, *Verrucomicrobiaceae* made up four. Three of the four OTUs within the family were suppressed by MSM and one promoted. *Verrucomicrobiaceae* are indicators for soil fertility and have been found to play important roles digesting organic matter and emitting carbon degrading enzymes (Ávila et al., 2019; Martinez-Garcia et al., 2012; Navarrete et al., 2015). *Planctomyces* were also suppressed by MSM; though they are a diverse genera, some have been connected to nitrogen immobilization (Buckley et al., 2015; Fuerst and Sagulenko, 2011). If carbon was promoting specialists, we would have expected both bacterial groups to be promoted, rather than suppressed.

Alternatively, if glucosinolate was a driving mechanism, we expected MSM to suppress pathogens and promote antimicrobial bacteria. MSM promoted the family *Rhodobacteraceae*, which has recently been found to release antimicrobial enzymes, including one that specifically reduces pathogenic bacteria (Henriksen et al., 2022; Murniasih et al., 2014). This single family is far from convincing proof of glucosinolate as a mechanism, but combined with reduced bacterial diversity, it does not rule out glucosinolate as an important factor affecting allelopathic connections between bacteria and weed suppression.

Interestingly, the family *Thermodesulfovibrionaceae* was promoted by MSM, though it's function or environmental preferences do not seem to add to the glucosinolate and carbon hypotheses. Some studies suggest that *Thermodesulfovibrionaceae* reduces sulfur and the family has been found in hydrothermal areas with high sulfur content (Arshad et al., 2017; Sonne-Hansen and Ahring, 1999; Sun et al., 2018). Brassica plants rely on sulfur for growth and exhibit improved seed and oil production with sulfur fertilization (Malhi et al., 2007).

Glucosinolate production is also positively correlated with higher soil sulfur contents (Borpatragohain et al., 2019). This suggests that mustard seed meal has a high sulfur content, which likely promoted sulfur reducing bacteria like *Thermodesulfovibrionaceae*.

Generally, relative abundance of almost all phyla were lower in MSM compared to control. This non-selective nature models the weed suppression patterns and supports the hypothesis that soil bacterial communities are an important mechanism for MSM weed suppression. This is an important aspect for growers to keep in mind before choosing to amend soils with MSM. Suppressed bacterial communities with lower diversity are associated with lower soil health – directly negating any efforts to improve soil health through cover crops or reduced herbicides and pesticides. Though an important factor to consider, there is much evidence that the soil microbiome quickly metabolizes both the carbon and glucosinolate from MSM, leading to short, but impactful effects on both weeds and soil health (Hannula et al., 2021; Hollister et al., 2013; Zaccardelli et al., 2013). Our study measured bacterial community composition 42 days after soil amendment, a relatively short time in terms of soil health, but longer than the microbial activity spike. Though unlikely to affect soil health within this timeframe, bacterial community composition differences could be harmful, or beneficial, to a newly planted cash crop. This study does not assess long-term bacterial community changes, and more research is necessary to assess MSM amendment effects on soil health and cash crops.

Conclusion

On average, MSM suppressed weed presence by 78% and biomass by 100%. The impartial selection does not support the occurrence of ecological theories including plant-soil feedbacks and nitrogen immobilization. Growers that want to take advantage of MSM as a natural alternative to herbicides should be aware of the non-selective nature and take precautions to avoid damage to their cash crops. MSM significantly increased microbial activity, but the effect spiked after seven days. This supports the fact that carbon from the MSM does stimulate the microbial community, and the impact subsides after the carbon resource has been depleted. Future field applications may benefit from this spike by carefully timing MSM amendment to kill weeds, but not the cash crop. Bacterial diversity was reduced by MSM, and bacterial community composition were dramatically different due to MSM. This supports the

hypothesis that the bacterial community plays a role in allelopathic relationships between MSM and weed suppression. Important OTUs were not supportive of the expected ecological responses to carbon, such as nitrogen immobilization. Glucosinolate likely plays a key role in the reduced diversity, though more detailed research is necessary to distinguish the confounded responses to carbon and glucosinolate. This work builds upon allelopathy and ecology literature specific to agriculture, an essential aspect to improve soil health management and agroecological practices.

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Figures and Tables

Figure 1

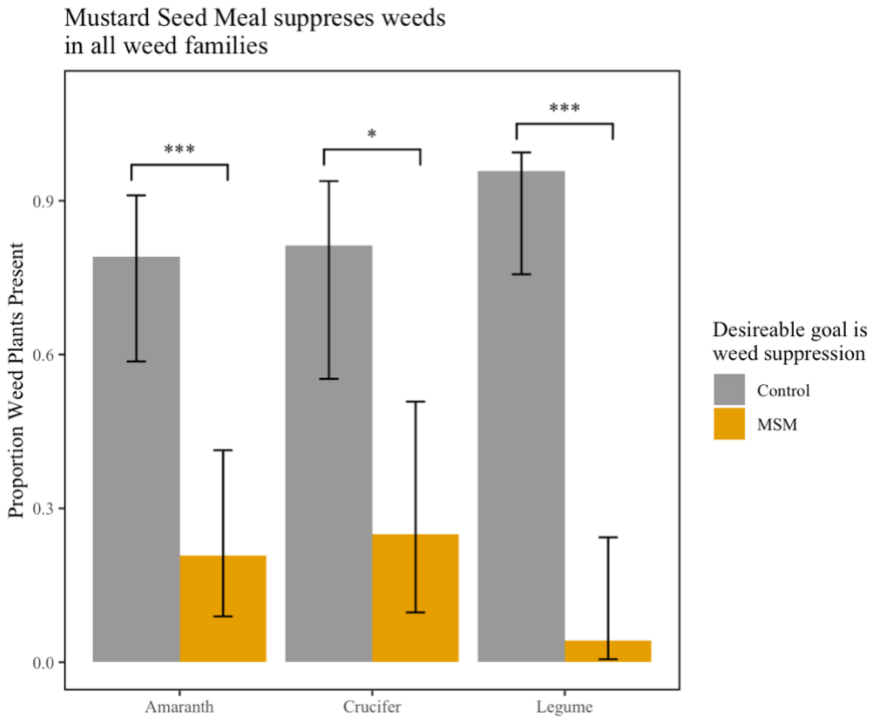

Figure 1: Mustard seed meal treatments (yellow) display significantly fewer plants present. The weed families were affected slightly differently, with weeds from the legume family affected more than Crucifer, but all other weeds affected the same.

Figure 2

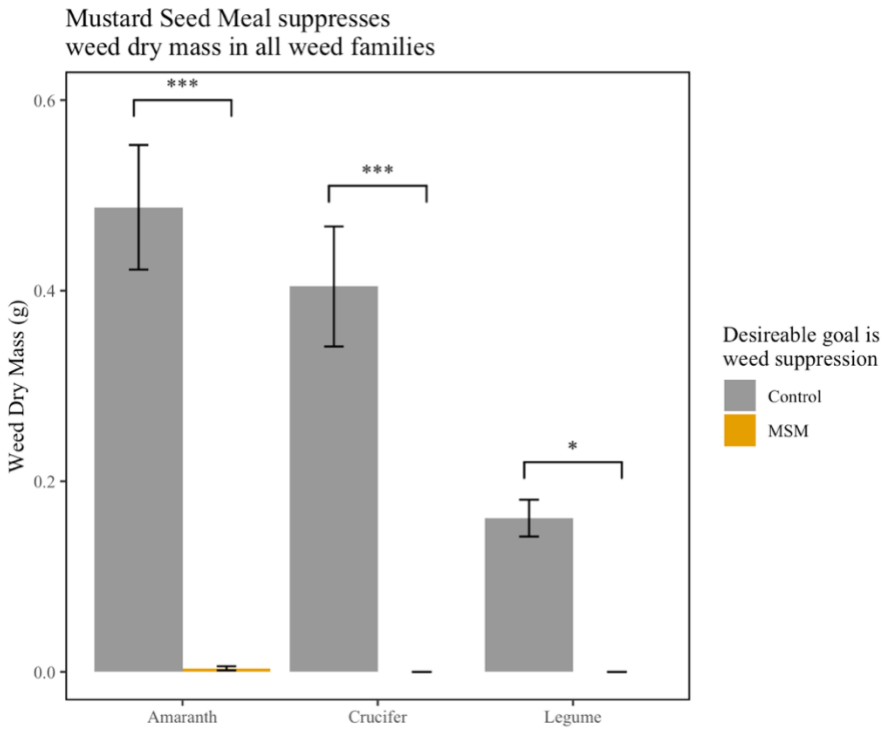

Figure 2: Mustard seed meal treatments (yellow) significantly reduced weed dry mass. Amaranth was affected the most, while Crucifer and Legume plants were affected equally.

Figure 3

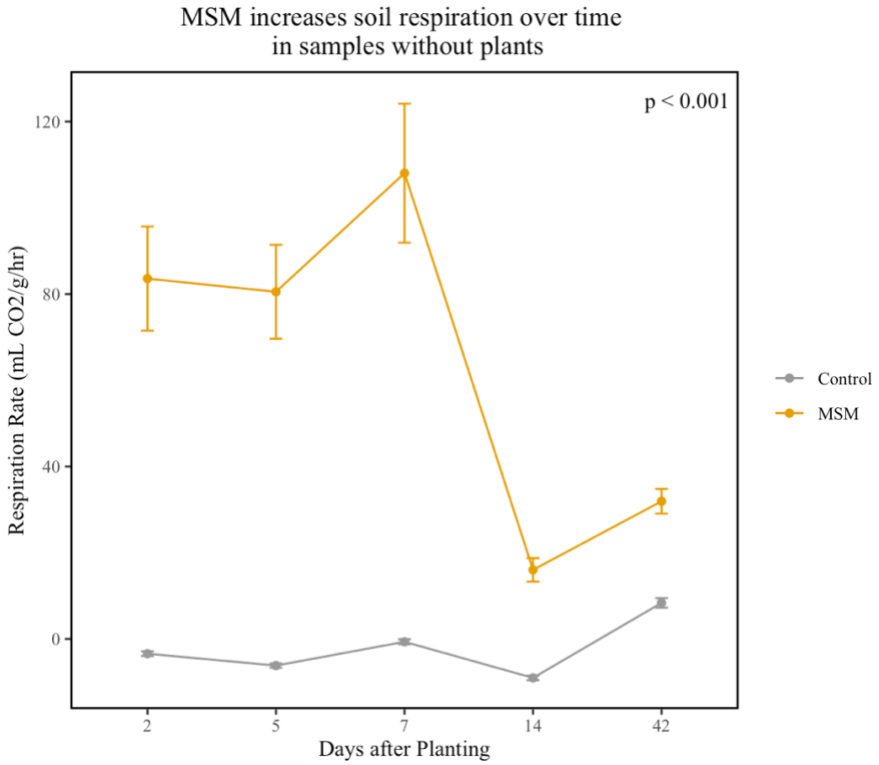

Figure 3: Soil respiration measurements were taken 2, 5, 7, 14, and 42 days after planting. MSM exhibited higher soil respiration across all measurements, though the soil respiration dramatically drops after seven days, likely due to microbes exhausting the carbon food source provided by the MSM.

Figure 4

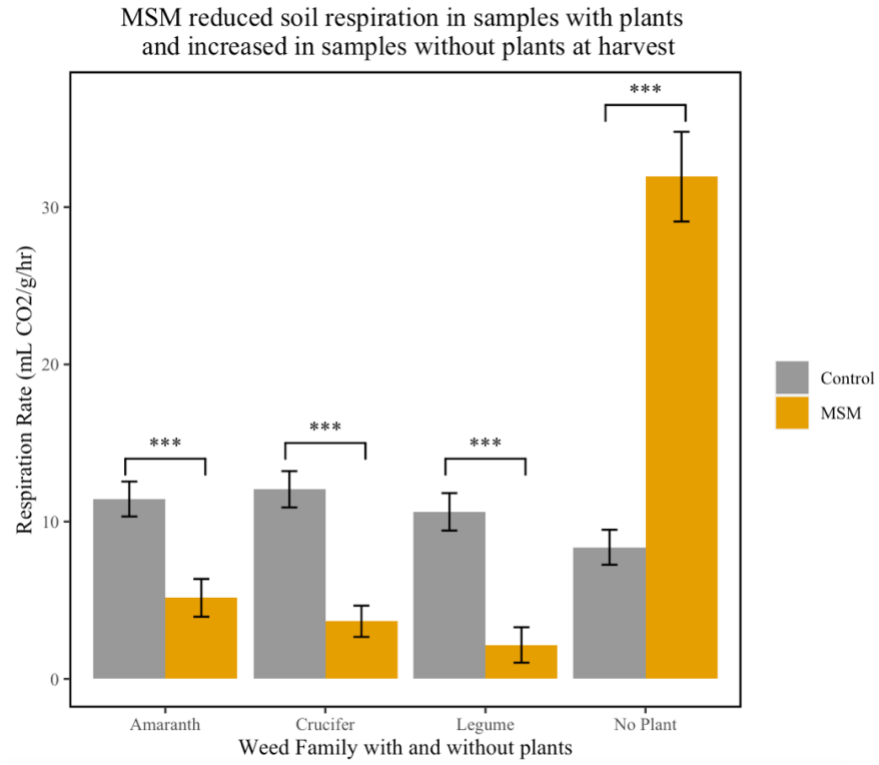

Figure 4: Mustard seed meal reduced soil respiration when plants were present but increased the soil respiration without plants. Since the MSM treatment so effectively reduced all weeds, it is possible that carbon from the plants in the control stimulated microbial activity, while the lack of plants in the MSM treatment resulted in the observed microbial activity reduction.

Figure 5

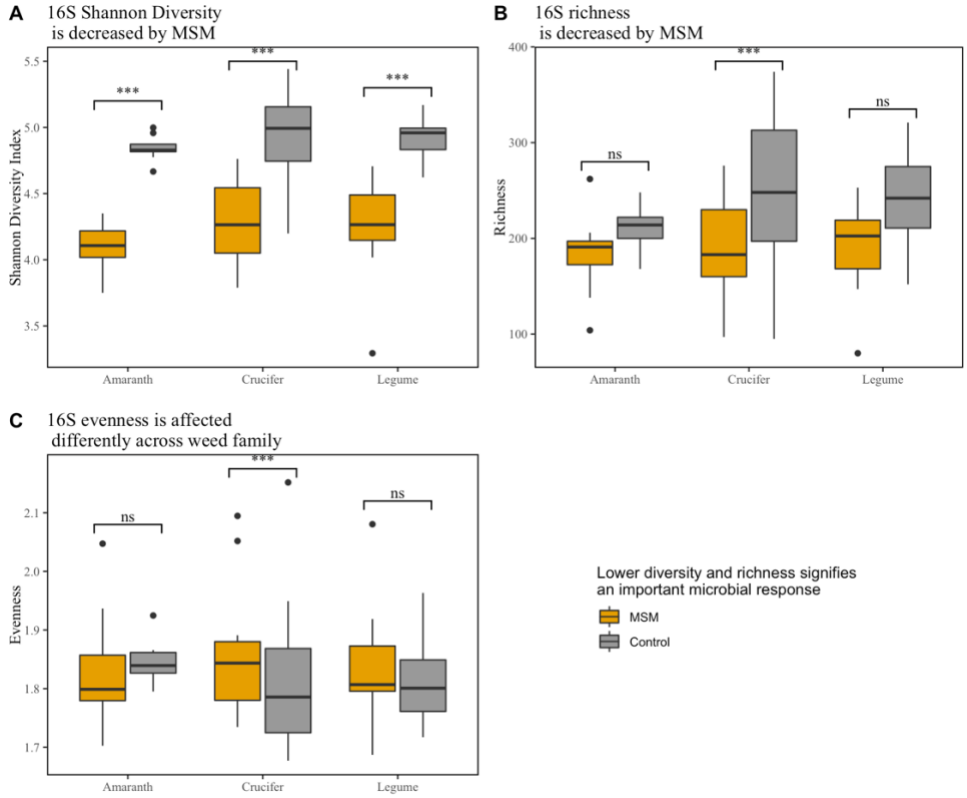

Figure 5: Mustard seed meal decreased bacterial community Shannon Diversity, evenness and richness were not significantly affected. Weed families were not affected differently.

Figure 6

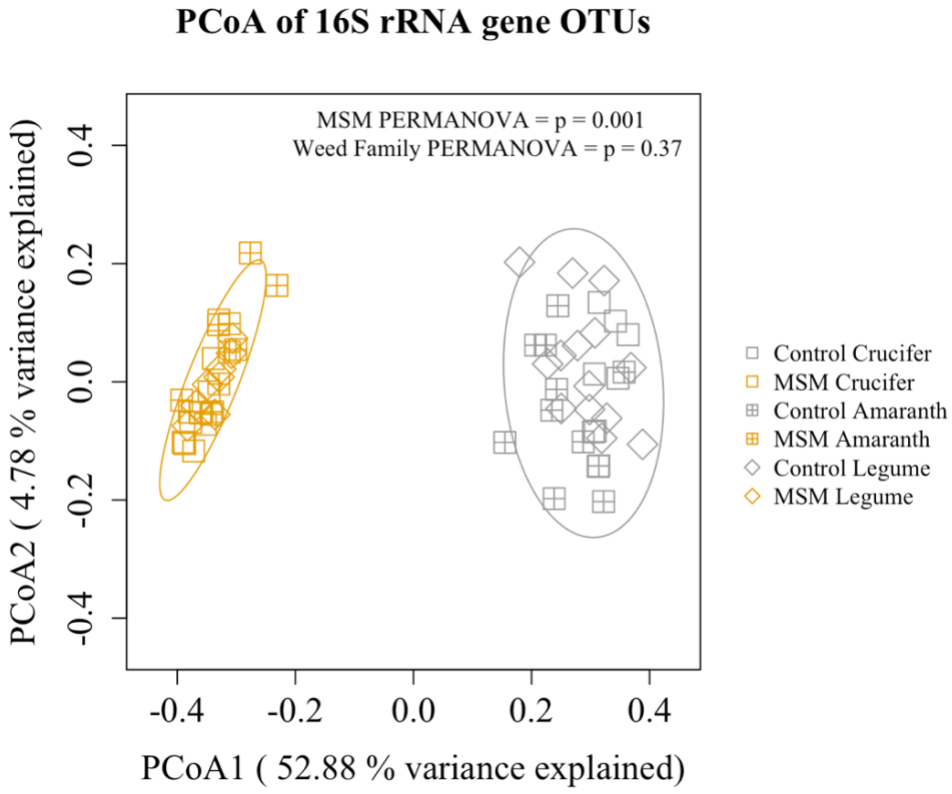

Figure 6: Bacterial community composition is significantly different between MSM and control, but not across weed families. Ellipses demonstrate the 95% confidence interval.

Figure 7

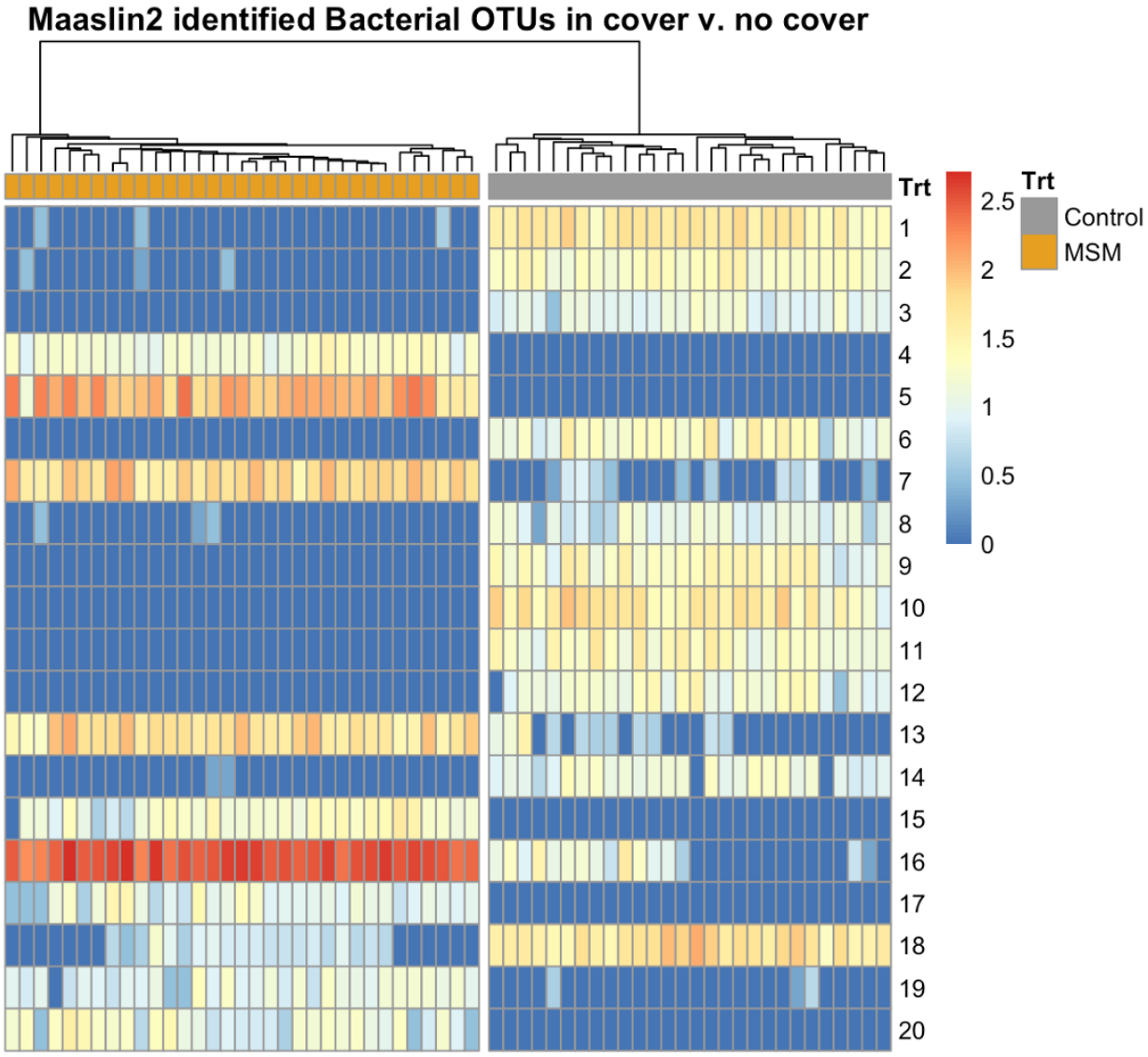

Figure 7: Maaslin2 identified OTUs that are most significantly different between treatments. Heatmap clustering confirms the difference with two distinct groups aligned exactly with the MSM and control. Relative abundance, displayed in blue to red tones shows which OTUs were suppressed or promoted in the MSM treatment. OTUs 1-3, 6, 8-12,14, and 18 were repressed, while the remaining were promoted. OTUs 2, 3, 4, 6, 9-13, and 17-19 were also identified by randomForest.

Table 1

Weed Functional Group	Weed Species	Weed plant reduction (%)	Weed biomass percent reduction (%)
Amaranth	Lambsquarters	-66.67*	-97.73*
	Palmer Amaranth	-100*	-100*
	Redroot Pigweed	-33.33	-100
Crucifer	Yellow Rocket	-66.22	-100*
	Shepherd's Purse	-71.42*	-100*
Legume	Black Medic	-100*	-100
	Red Clover	-87.5*	-100*
	White Clover	-100*	-100

Table 1: Mustard seed meal reduced weed plant presence and weed biomass of all species, though some like Yellow Rocket and Redroot Pigweed were not significantly reduced.

Table 2

Top 20 Bacterial OTUs Identified by randomForest					
#	**Phylum**	**Class**	**Order**	**Family**	**Genus**
1	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	Helicobacter
2*	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	Helicobacter
3					
4					
5*	Proteobacteria	Deltaproteobacteria			
6*	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	
7*	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	
8	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	
9*	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	
10*	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	Helicobacter
11*					
12*	Nitrospirae	Nitrospira	Nitrospirales	Thermodesulf-ovibrionaceae	BD2-6
13*					
14	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Planctomyces
15					
16*	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	
17*					
18*	Proteobacteria				
19	TM6	SJA-4			
20	Nitrospirae	Nitrospira	Nitrospirales	Thermodesulf-ovibrionaceae	BD2-6

Table 2: The top 20 Bacterial OTUs identified by randomForest act as predictive for either MSM or control. For example, if one were to randomly sample from a section of the field, without know which treatment you sampled from, randomForest would be able to use these OTUs to identify which treatment the sample came from with 0% error rate. OTUs that were also identified by Maaslin2 are denoted with an asterisk.

Table 3

Top 20 Bacterial OTUs Identified by Maaslin2					
#	**Phylum**	**Class**	**Order**	**Family**	**Genus**
1	Proteobacteria				
2*	Proteobacteria				
3*					
4*	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	
5	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	Helicobacter
6*	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	
7	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	Helicobacter
8					
9*	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	
10*	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	
11*	Proteobacteria	Deltaproteobacteria			
12*	Proteobacteria				
13*					
14	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	
15					
16	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Rhodoplanes
17*	Nitrospirae	Nitrospira	Nitrospirales	Thermodesulf-ovibrionaceae	BD2-6
18*	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Planctomyces
19*	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	
20	Nitrospirae	Nitrospira	Nitrospirales	Thermodesulf-ovibrionaceae	BD2-6

Table 3: The top 20 most significantly different OTUs between MSM and control, identified by Maaslin2. Maaslin2 is another way to evaluate OTUs that serve as important distinctions between treatments. 12 of the OTUs identified by randomForest were also identified by Maaslin2, marked with asterisks.

Supplementary Figures

Figure 8

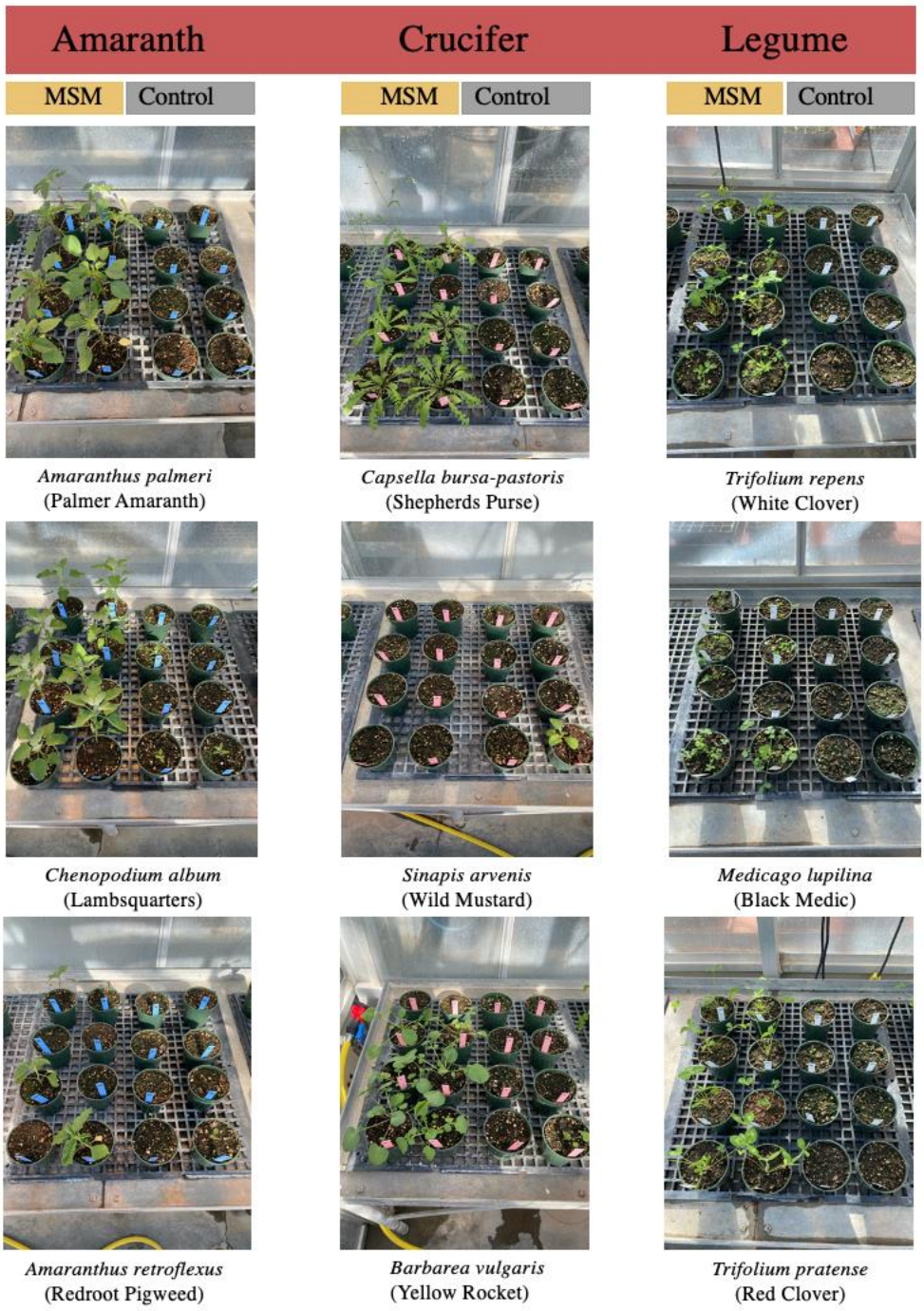

Figure 8: Pictures of weed growth at the end of harvest show the dramatic effects due to MSM. Amaranth family weeds are in the left-hand column, Crucifer in the center, and Legume in the right-hand column. In each picture, the control treatment is the two columns of pots on the left, and the MSM treatment is the two columns of pots on the right.

Figure 9

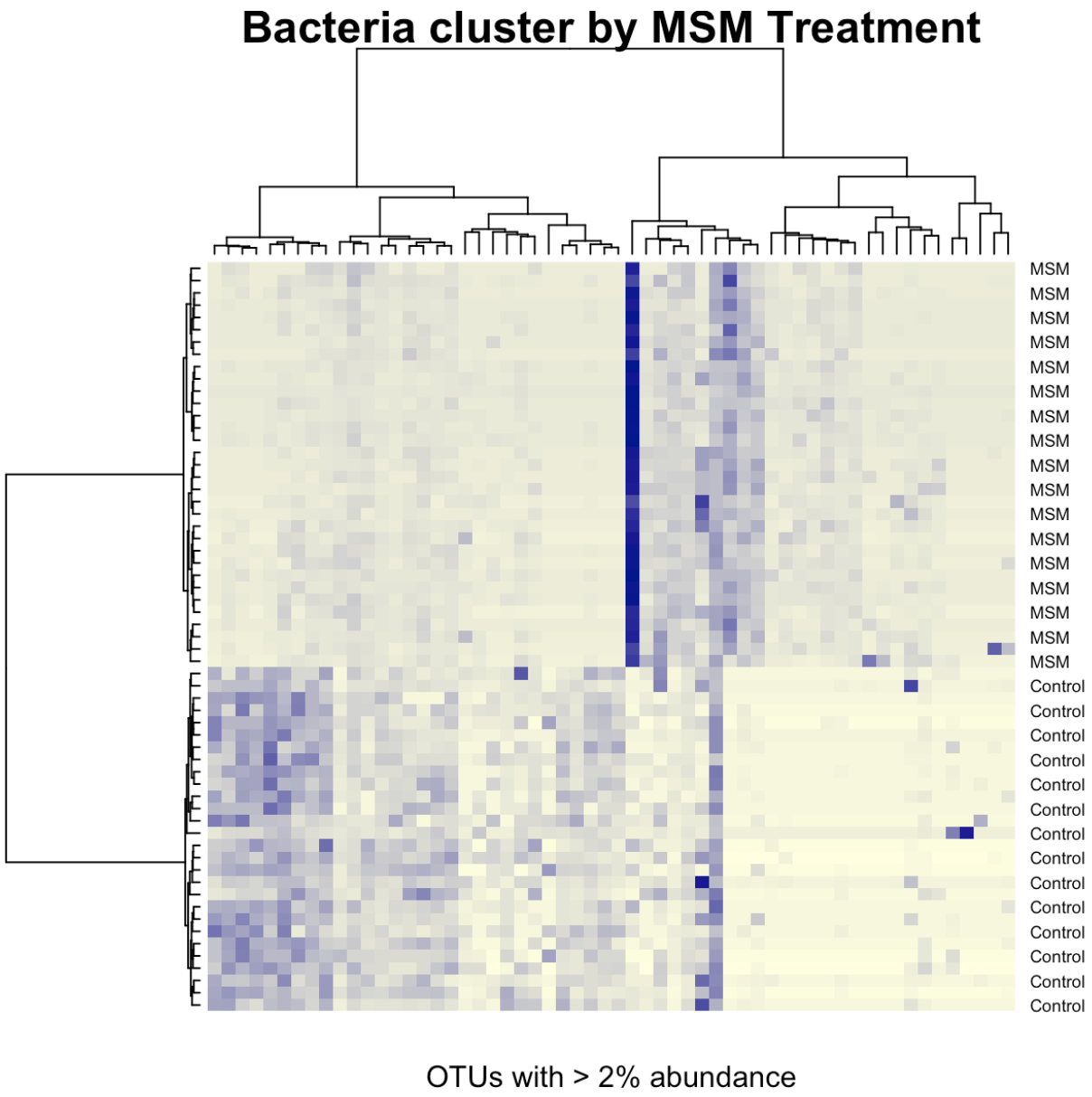

Figure 9: Heatmap clustering of OTUs with more than 2% relative abundance supports PCoA and PERMANOVA results. The OTUs are distinctly clustered into two groups, as seen in the phylogenetic trees along the left-hand side. These two groups perfectly match MSM and control samples, as seen along the right-hand side.