UNDERSTANDING THE IMPACT OF FUNGICIDES ON LOWBUSH BLUEBERRY SOIL ECOLOGY THROUGH MICROBIOME METHODS

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ABSTRACT

Lowbush blueberries are an economically significant crop produced by managing naturally occurring populations. As such, they have an intimate relationship with the soil microbiome and depend on it for their health and productivity. Fungicides are important to reduce disease pressure but pose a potential risk to soil health. Metagenomic methods are a powerful set of tools to understand the nature of these plant-microbiome interactions and the ways in which fungicides affect them. This thesis contains the findings of two studies comparing fungal and bacterial populations of soils treated with fungicides to those of untreated soils, using amplicon sequencing. In both, significant changes to the soil ecosystem were found as a result of fungicide treatment. While the specific findings of the studies differed, their combined results suggest that changes to soil ecosystems occur due to fungicide application, and that further research is needed to understand its long-term effects on soil health.

LIST OF ABBREVIATIONS USED

AM	Arbuscular Mycorrhizal
ASV	Assigned Sequence Variant
CB	Cabrio
CTG	Combined Treatment Group
EC	Enzyme Commission
EcM	Ectomycorrhizal
ErM	Ericoid Mycorrhizal
FRAC	Fungicide Resistance Action Commission
ITS2	Internal Transcribed Spacer 2
LT	Luna Tranquility
MB	Miravis Bold
ME	Merivon
MP	Miravis Prime
PCA	Principal component analysis
PCoA	Principal coordinates analysis
SC	Scala
SL	Scholar
SR	Sercadis
UTG	Untreated Group
VP	Velum Prime

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CHAPTER 1: INTRODUCTION

Microbe-plant interactions play a critical role in the agroecological system, and understanding these relationships is a significant frontier in plant cultivation and pathology. Plant-microbe symbiotic relationships are the product of concurrent evolution between plants and microbes and are, in many cases, necessary to ensure that the plant will thrive (Zilber-Rosenberg and Rosenberg 2008). These interactions may be particularly important in case of lowbush blueberries which are grown as a managed wild crop throughout the Atlantic Provinces of Canada, Quebec, Maine and New Hampshire. Lowbush blueberries are primarily managed as a crop in Atlantic Canada, as well as Quebec, New Hampshire and Maine (Strik and Yarborough 2005). In terms of total cropland, blueberries are the most widely grown crop in Canada and have a total export value of around $410 million (Agriculture and Agri-Food Canada 2019). Wild blueberry production is an attractive model to study the interaction of the microbiome, plant and environment because this system involves transformation of the environments surrounding the plant to intensify plant propagation and yield (Hall et al. 1979; Eaton 1988; Drummond et al. 2009; Bell et al. 2009). As a result, the plants native habitats remain unchanged in wild blueberry production system. Therefore, this system is an advantageous model to study the effects of environmental factors on soil and plant-associated microbiomes, the interaction between soil microorganisms and host-plant, and the functional differentiation of soil and plant-associated microbiomes (Yurgel et al. 2019).

Lowbush blueberries occur naturally on sandy acidic soils in their native range. Commercial production of lowbush blueberries consists of managing wild populations through practices such as weed and pest control, fertilization, commercial pollination, and pruning to establish an area as a lowbush blueberry production field (Drummond 2012; Esau et al. 2015, 2019). Typically, a lowbush blueberry field is heterogeneous in its taxonomic composition with a mixture of *Vaccinium angustifolium* and *Vaccinium myrtilloides* (Drummond et al. 2009). The natural habitat of the wild blueberry is the sandy, nutrient-poor soil of the forest understory (Drummond et al. 2009). About 70% of the plant's biomass is subterranean, with a correspondingly small proportion of the plant growing above the soil. This species establishes extensive rhizome networks, from aerial parts of the plant grow (Yarborough 2012). As a result, the plants are highly capable of regenerating after losing their aerial biomass (Drummond et al. 2009). A comprehensive rhizome network is crucial to establishing a commercial blueberry field and remains a significant impediment to lowbush blueberry production outside of their native range (Yarborough 2012). While, the roots of the plant from the family Ericaceae are notably thin and lacking of root hairs (Smith and Read 2008), the lowbush blueberry establish extensive networks of rhizomes, modified stems which grow horizontally under the soil's surface, from which new, aerial stems grow (Yarborough 2012). As a result, the lowbush blueberry is exceptionally capable of regenerating after a complete loss of its aerial biomass (Drummond et al. 2009). A comprehensive rhizome network is a necessary aspect of a successful commercial blueberry field and is the primary obstacle to the

establishment of lowbush blueberry crops outside of their native range (Yarborough 2012).

CHAPTER 2: LITERATURE REVIEW

2.1 Plant-beneficial microbiome

The plant-associated soil ecosystem can be described in terms of several sub-habitats. The microorganisms existing in the closest associations with the plant are the endophytes, those which colonize plant tissues, including those of the root (Vandenkoornhuyse et al. 2015). These may include fungal symbionts, notably the arbuscular mycorrhizal fungi (AM fungi), and the ericoid mycorrhizal (ErM) fungi, as well as bacterial endophytes (Smith and Read 2008; Martínez-Romero 2009; Vandenkoornhuyse et al. 2015). Moving outwards from the root endosphere, the rhizoplane (the surface of the root), is another significant realm of plant-microbe association and is the primary site of ectomycorrhizal (EcM) fungal colonization of woody plants (Smith and Read 2008; Vandenkoornhuyse et al. 2015). The layer of soil most adjacent to the root surface, the rhizosphere, is also an important area of chemical exchange between microbes and the plant and is a significant site of both pathogen and symbiont recruitment. Finally, the bulk soil serves as the repository of microorganisms and their spores from which the root microbiome is recruited (Vandenkoornhuyse et al. 2015). All of these microorganisms form an extended ecosystem surrounding the plant, with a network of interactions between the plant and the microbes, and between the microbes themselves (Vandenkoornhuyse et al. 2015). In keeping with this notion, it has been demonstrated that the microbiomes surrounding *Vaccinium* plants differ significantly, not only at the species level, but also at the intraspecies genotypic level (Li et al. 2020). As a result, plants, in addition to relying on the

microbiome in which they are situated, create, through their presence, a microbiome best suited to their needs.

2.1.1 Fungi

Microbial relationships are crucial to plant survival. Mycorrhizal associations, in particular have been well-studied for their role in plant nutrient acquisition. Ericoid mycorrhizal (ErM) fungi enable Ericaceous plants to absorb nitrogen from an otherwise nitrogen-poor environment (Mitchell and Gibson 2006). EcM fungi have been demonstrated to fill a critical role in the acquisition of phosphorous by woody plants in the forest ecosystem (Cairney 2011). However, these symbiotic relationships perform more services than mere nutrient acquisition. For instance, AM fungi have been shown to contribute to the suppression of plant disease and help plants to better tolerate abiotic stresses such as drought and salinity (Veresoglou and Rillig 2012; Moradtalab et al. 2019; Ait-El-Mokhtar et al. 2019 p. 0).

Plants in the genus *Vaccinium*, as many members of order Ericales, develop a distinctive mycorrhizal relationship with ErM fungi (Smith and Read 2008; Brundrett and Tedersoo 2018; Yang et al. 2018a). ErM is one of the primary reasons that lowbush blueberries are able to thrive in the harsh, nutrient-poor soils with which they are associated. ErM fungi grant the plant access to organic sources of nitrogen in the soil which would otherwise be unavailable to them (Mitchell and Gibson 2006). While typically plant uptake of nitrogen is confined to nitrogen in simple inorganic forms (such as NO_3 and NH_4), plants of genus *Vaccinium* which had been inoculated with ErM fungi have been

demonstrated to be able to uptake and metabolize amino acids from the soil, while non-inoculated plants of the same species were unable to do the same (Stribley and Read 1980). ErM fungi have also been shown to be capable of using nitrogen from longer peptide chains as well, making these more complex forms of nitrogen available to their plant hosts (Bajwa and Read 1985; Mitchell and Gibson 2006). For instance, one ErM fungi, *Oidiodendron maius*, contains genes which encode for enzymes capable of degrading the necessary polysaccharides to break down the cell-walls of sphagnum moss (Tsuneda et al. 2001; Kohler et al. 2015). The idea that blueberries are heavily dependent on organic nitrogen sources is further bolstered by the fact that, compared to some of its weeds, the lowbush blueberry is significantly less efficient at absorbing nitrogen from inorganic sources (Marty et al. 2019a).

The taxonomic identities of ErM fungi contain many ambiguities, however, the group is known to be taxonomically diverse and to contain both Ascomycetes and Basidiomycetes (Martino et al. 2018). Where Basidiomycetes are concerned, it has been shown that members of the order Sebacinales commonly formed mycorrhizal associations with plants of the family Ericaceae (Selosse et al. 2007). Another Basidiomycetes group which has been more controversially identified as ErM fungi is the genus *Clavaria* (family Clavariaceae), members of which have been demonstrated to take part in a transference of nutrients between the fungi and ericaceous plants (Englander and Hull 1980). Additionally, sequencing data has demonstrated that *Clavaria* were found of the ErM communities in *Vaccinium uliginosum*, lending further credence to the notion that *Clavaria* form ErM relationships (Yang et al. 2018a). Perhaps the most well-studied ErM

fungal taxa is *Rhizoscyphus ericae* (formerly *Hymenoscyphus ericae*), part of what has been called the *H. ericae* aggregate, a group of closely related fungal taxa which perform different types of symbiosis. (Vrålstad et al. 2002; Smith and Read 2008). Additionally, it has been noted that there is taxonomic overlap between those fungi which form ErM and those which form ectomycorrhizae, with ErM species being shown to form endophytes Norway spruce roots (Vohník et al. 2013).

Metagenome approaches to lowbush blueberry rhizosphere and endophyte communities showed an increased relative abundances of Leotiomycetes taxa, such as putative ericoid mycorrhizal fungi Lachnum and potential plant growth promoting dark septate endophyte Phialocephala, as well as lichen-forming fungi Lecanorales, *Cetradonia linearis* and Cladonia in wild blueberry root microbiome. Additionally, Leotiomycetes (containing many plant pathogens and mycorrhizal fungi) were identified as one of the most influential (hub) taxa in root-associates microbiome interaction network (Yurgel et al. 2018b). These hub taxa could directly and indirectly influence microbial communities by inhibiting or facilitating the growth of other microbes affecting overall interconnected communities, as well as by affecting the host-plant and triggering the changes in plant species sorting preferences (Agler et al. 2016). In another study, Leotiomycetes Helotiales, which contains several known ErM species, was found to be the most abundant fungal order in the lowbush blueberry rhizosphere. In particular, two species, *Pezoloma ericae* and *Oidiodendron maius* (known to form ErM), were found to be highly abundant (Morvan et al. 2020). Leotiomycetes *Phialocephala* was another highly abundant genera found in the lowbush blueberry rhizosphere. This taxa contains several species known to be dark

septate endophytes, which have been shown to colonize European blueberry roots in a manner superficially similar to ErM while not performing the functions of ErM (Lukešová et al. 2015; Morvan et al. 2020). Additionally, the presence of Helotiales species, such as *Pezoloma ericae*, *Meliniomyces bicolor*, *Oidiodendron chlamydosporicum* and *Philocephala fortinii*, was linked to an increased leaf nutrient content in Vaccinium angustifolium suggesting a beneficial effect of these fungi on the host-plant (Morvan et al. 2020).

2.1.2 Bacteria

In additional to fungal symbionts, some bacterial associations with lowbush blueberries have been identified by 16S amplicon and metagenome sequencing. Overall, Alphaproteobacteria, Gammaproteobacteria, Actinobacteria, and Bacteroidetes were the most abundant bacterial taxa in wild blueberry roots and were enriched along the soil-endosphere continuum (Yurgel et al. 2017, 2018b). More specifically Rhizobiales was found to be the most abundant bacterial order in the rhizospheres of managed lowbush blueberry, and *Bradyrhizobium* was the most abundant genera overall. Interestingly, it was found that the presence of *Bradyrhizobium* corresponded to higher concentrations of nitrogen in plant leaves (Morvan et al. 2020), which was probably linked to the ability of some *Bradyrhizobium* spp to fix atmospheric nitrogen inside plant tissues. In addition to ability for nitrogen fixation *Bradyrhizobium* spp. are able to promote plant growth by producing the plant growth promoting compounds indole-3-acetic acid and 1-amino-cyclopropane-1-carboxylic acid deaminase (Piromyou et al. 2017). *Bradyrhizobium* and

Pedosphaerales, were also hubs in the network considering root-associates wild blueberry microbiome (Piromyou et al. 2017; Yurgel et al. 2018a)**.**

2.1.3 Function

The functional characteristics of the wild blueberry bulk soil and rhizosphere microbiome investigated with shotgun metagenomics sequencing identified high relative abundances of potential nitrogen and carbon fixating microorganisms suggesting an adaptation of the microbiome to low fertility soils typical for wild blueberry habitats (Yurgel et al. 2019)**.** Wild blueberry exhibited lower relative abundance in general metabolic functions and higher relative abundance in sugar and putrescine transport, as well as degradation of complex organic compounds. It was proposed that overrepresentation of pathways involved in biodegradation xenobiotics and terpenoids in rhizosphere could provide the microbiome with functional flexibility to respond to plant stress status (Yurgel et al. 2019).

2.2 Lowbush blueberry pathogens

Fungal pathogens are the most significant pathogens of lowbush blueberries, in terms of disease pressure and prevalence. *Monilinia vaccinii-corymbosi*, the pathogen responsible for the mummy berry disease, is one of the most significant pathogens of lowbush blueberries. Infections of the fruit lead to desiccated berries (Milholland 1977). A particularly significant disease risk in fields with heavy soil and limited air flow (Percival and Beaton 2012). Primary infection occurs via ascospores on vegetative tissues, followed by secondary infection of flowers via conidiospores. Highly specialized, the pathogen uses

the plants pollen tubes to begin hyphal growth for secondary infection (Ngugi and Scherm 2004; Lehman et al. 2007). Another severe crop-year pathogen of lowbush blueberries is *Botrytis cinerea*, which leads to the disease Botrytis blossom blight. The genus *Botrytis* has an extremely broad host range, infecting over 1400 plant species, many of which are significant agricultural crops, and it is considered to be among the most important of all plant pathogens (Dean et al. 2012; Elad et al. 2016). Furthering *Botrytis'* status as a pernicious generalist is its capacity to feed as both a pathogen and a saprotroph, to varying extents depending on the strain (Martinez et al. 2003). For lowbush blueberries, their floral nodes are most susceptible to *Botrytis* infection immediately after blooming. The conditions which favor the spread of *Botrytis* are temperatures between 16 and 24 degrees with high humidity, with a film of water being a factor which confers an advantage to the pathogen (Hildebrand et al. 2001; Elad et al. 2007). Analysis of the wild blueberry microbiome indicated accumulation of Leotiomycetes, the class containing *Monilinia vaccinii-corymbosi* and *Botrytis cinerea*, in plant roots (Yurgel et al. 2018a)**.** *Septoria* represents another significant risk to blueberry crops, producing lesions on leaf surfaces which ultimately constrict the amount of photosynthesis the plant can perform and thus limit the amount of carbohydrates it can store up for producing floral nodes in the fall (Roloff et al. 2004).

2.3 Soil health and microbial communities

Given the critical roles played by microbial communities in plant health and production, evaluating soil health ought to include an understanding of the microbiome. Soil health refers to the biological processes of the soil and its capacity to carry out

ecosystem services (Lal 2016). Indeed, these ecosystem services extend beyond direct symbiosis, with microorganisms playing key roles in cycling macronutrients (Dubey et al. 2019). As a result, understanding the microbial community will lead to better-informed choices in agricultural management. Indeed, it was found that microbial community parameters strengthen predictions of environmental processes, with 53% of soil respiration models being improved by microbial community data (Graham et al. 2016). Additionally, in agricultural soils in particular, microbial factors were stronger predictors of environmental processes relative to non-agricultural soils (Graham et al. 2016).

Soil fertility is one of the important parameters of agricultural soils. While in general wild blueberry habitats characterized by low soil fertility, Nova Scotian soil in wild blueberry production fields differ in their chemical composition. This is reflected in variations in total nitrogen, organic matter, cation-exchange capacity as well as sulfur, phosphate content. Based on these characteristics the soils from wild blueberry managed habitats can be separated on to cluster, high and low fertility soils (Yurgel et al 2017). The higher fertile soils were overpopulated with Fungi, Ascomycota, Basidiomycota, and Mucoromycota, Metazoa/Rotifera, Archaeplastida/Embryophyceae, and Rhizaria/Filosa-Sarcomonadea, while the low fertile soils were characterised by increased relative abundances of Prymnesiophyceae, Telonemia, Urochordata, and Acantharea. Additionally, high soil fertility was correlated with increased eukaryotic alpha-diversity (Yurgel et al. 2017).

Management can significantly influence soil health parameters including soil biology. Because of the uniqueness of wild blueberry production system, it provides a

powerful baseline for evaluating plant-microbiome responses to relatively minor land-use changes. It was shown that the aggregate difference in forest vs. managed systems was not a strong factor affecting eukaryotic communities from both bulk and rhizosphere soils, while it had a strong effluence on soil and rhizosphere bacteria. It was shown the managed soils exhibited a decrease in relative abundances of arbuscular mycorrhiza and increase in relative abundance of fungal pathogens. Moreover bacterial communities from managed field were more diverse compared with forest soils (Yurgel et al. 2017). Comparison between soil microbiomes from the filed with high and low crop production also identified several eucaryotic taxa linked to decreased plant yield. Parasitic fungi Cryptomycotina, gliding bacterivores/algaevores Glissomonadida and Vampyrellida were detected in higher relative abundances in the fields with low fruit yield compared to that from fields with high fruit yield. The overrepresentation of Cryptomycotina might affect plant health through the disease development, while the high relative abundance of Glissomonadida and Vampyrellida can result in depletion of microbial taxa with beneficial effect on plant development and production (Yurgel et al. 2017). However, more detailed studies are required to verify this hypothesis. Other research, however, suggests that not all forms of management have significant effects on the soil microbiome. Research into the effects of differing types of mulch on the soil microbiome of the highbush blueberry (*Vaccinium corymbosum*), with a focus on plant growth promoting bacteria, found that no significant differences to the microbial community could be attributed to mulch (Lee et al. 2021).

2.4 Fungicides and the microbiome

The question of the effects of pesticides on the health of the soil microbiome is one of considerable environmental concern and has been extensively studied. However, given the multiplicity of pesticides, both in terms of target organism (insecticide, fungicide, herbicide, etc.) and active ingredient mode of action, mobility, and persistence, it can be difficult to predict the effects of a given pest control product to a soil's ecosystem. For instance, fungicides have been linked to an increase in soil organic matter and, as a result, microbial activity (Pagano et al. 2017). Conversely, the effect of fungicides on the health of the soil microbiome tends to be deleterious. The application of many fungicides resulted in decreased levels of soil biological carbon and nitrogen, and had variable effects on the nitrogen cycle depending on the environmental context of the experiment (Ullah and Dijkstra 2019).

The application of fungicides has been found to have significant effects on wild blueberries soil microbiome, with a reduction in fungal species richness in fungicide treated soils. Additionally, fungal community structure was significantly altered by the presence of fungicides (Lloyd et al. 2021). While bacterial species richness and community structure did not show significant changes, the predicted function of the bacterial microbiome did present some notable differences between treated and untreated plots. Most notably, a number of enzymes associated with the degradation of fungicides had increased in relative abundance, suggesting a bacterial role in the breakdown of fungicides (Lloyd et al. 2021). The increased relative abundance of these enzymes may indicate a selection process through which pesticide-degrading bacteria become more

prevalent. This shift in community composition may in turn lead to a more rapid breakdown of pesticide compounds and may, ultimately, reduce the efficacy of the compounds in use. It has been shown that repeat applications of a pesticide may lead to an accumulation of these traits in a bacterial population and thus progressively diminish the chemical's ability to control pathogens over time (Arbeli and Fuentes 2007). One possible remedy to the problem of bacterial biodegradation is to combine chemical fungicides with biological fungicides (principally composed of *Bacillus* spp.). These biological agents have been shown to provide significant protection from fungal disease and, when used in rotation with synthetic fungicides, provide comparable protection to synthetic fungicides alone (Abbey et al. 2020). Biological fungicides, by not providing a carbonaceous substrate to promote the growth of biodegradation of fungicides, may thus help to prevent the accumulation of biodegrading bacteria which reduce the effectiveness of synthetic fungicides.

One fungicide in particular, prothioconazole, was associated with a significant increase in the relative abundance of the fungal family Clavariaceae and one of its constituent species, *Clavaria sphagnicola*. This finding may be noteworthy in this context as *C. sphagnicola* and other taxa in genus *Clavaria* have been associated with the formation of ErM with plants in the genus *Vaccinium* (Yang et al. 2018a; Lloyd et al. 2021). If *C. sphagnicola* or other taxa from this family are forming ErM with lowbush blueberries, their increased relative abundance may be the result of improved plant health as a result of decreased disease pressure. In this case, a more productive plant population would have a greater abundance of resources with which to maintain a larger symbiont

population. Or, in a similar scenario, it may be that the healthier plant population produced more extensive root growth which, in turn, lead to greater opportunities for mycorrhizal symbiosis. As a result, the increased relative abundance of these taxa may be a positive indicator of plant health in the presence of prothioconazole.

If the causality of the situation is reversed, and prothioconazole lead directly to an increase in the relative abundance of *Clavaria* species acting as symbionts to the plant, it may indicate a beneficial effect to plant fitness through that factor alone. Increasing growth of arbuscular mycorrhizal fungi has been observed in some contexts when exposed to select fungicides (von Alten et al. 1993). These observations may imply that a similar increase in relative abundance of symbiotic fungi in response to prothioconazole may be plausible. In such a situation the fungicide would be providing benefit to the plant through more than just the suppression of disease pressure but would also be improving plant fitness through strengthening populations of beneficial mycorrhizal fungi.

2.5 Microbiome Sequencing

To study the microbiome, genomic sequencing methods are used to create a profile of the microbial ecosystem at the time of sampling. To do so, samples of the ecosystem to be examined, such as soil or water samples, are first taken. Then, genetic material is extracted by lysing the microbial cells present and isolating the DNA from its surrounding chemical substrate (Roose-Amsaleg et al. 2001). Using this DNA, the target genetic material is amplified using PCR-primers corresponding to the desired genetic region (Poretsky et al. 2014). This amplified genetic material is sequenced via a high-throughput sequencing platform, such as Illumina MiSeq (Comeau et al. 2017). The final

step of the process is bioinformatics, through which the sequences are filtered for quality and assigned taxonomies by comparison to databases of known microbial sequences (Quast et al. 2013; Rognes et al. 2016; Amir et al. 2017). The bioinformatics process also enables the calculation of qualitative and quantitative metrics through which levels of diversity, community structure, relative abundances of taxa, and phylogenetic relationships between taxa can be assessed.

2.5.1 Amplicon Sequencing

Amplicon sequencing, or marker gene sequencing, consists of sequencing a relatively small genetic region, from which the taxonomic abundances of organisms in the microbiome can be inferred (Woese 1987; Hamady and Knight 2009; McLaren et al. 2019). These taxonomic abundances, when used in combination with previously-established information about the potential microbial function, can be used to predict the microbial functions being performed in the sample ecosystem (Douglas et al. 2020). However, as amplicon sequencing does not involve the sequencing of entire genome, it cannot be used to discover new functions in the microbiome and necessarily relies on previously understood functions. Regardless, amplicon sequencing is a cost-effective method of microbiome analysis with well-established workflows and comprehensive gene databases (Quast et al. 2013; Ranjan et al. 2016; Comeau et al. 2017).

2.5.2 16S rRNA gene

The 16S rRNA gene, the preeminent amplicon gene used to identify prokaryotic organisms, encodes for a unit of ribosomal RNA (rRNA) which forms part of the 30S ribosomal subunit in prokaryotes (Srinivasan et al. 2015). The 16S rRNA gene contains

both highly conserved and highly variable segments, making it a consistent measure of phylogenetic relations between prokaryotes (Woese 1987). By referencing sample genes isolated from the study environment against a database of 16S sequences, it is possible to accurately assign taxonomic identifications to the microbiome (Srinivasan et al. 2015). This method of identifying microorganisms has been verified against culture-based clinical identification, with 16S rRNA sequencing being shown to have a success rate of 96% at identifying taxa at the genus level (Srinivasan et al. 2015).

2.5.3 ITS and ITS2

Internal transcribed spacer, (ITS) is a region of ribosomal RNA cistron DNA and is one of the primary marker genes used in fungal amplicon studies. The ITS region is between 300bp and 1200bp in length and separates the 18S small rRNA subunit gene from the 28S large rRNA subunit gene (Heeger et al. 2019). Like the 16S rRNA gene, the ITS region varies significantly between species, has conserved primer sites, and is typically present in multiple copies (Blaalid et al. 2013). In a study of six potential genetic markers for identifying fungal taxa, ITS was determined to have the best combination of sequencing reliability and identification accuracy (Schoch et al. 2012).

The ITS region is divided into three sections, ITS1, ITS2, and the intercalary 5.8S region. Given the length of the ITS region, many sequencing methods require the ITS1 and ITS2 sections to be analysed separately (Blaalid et al. 2013). Though the two regions have comparable performance in taxonomic identification, the selection of ITS1 v. ITS2 may lead to biases in the taxonomic assignment of sequences (Blaalid et al. 2013). Of the two, identifications made from ITS2 reads have been found to more closely match full those of

full-strand ITS relative to ITS1 (Yang et al. 2018b). Additionally, ITS2 has been found to outperform 18S rRNA, and 26S rRNA regions in identifying fungi in the human mycobiome (Hoggard et al. 2018).

2.6 Conclusion

The plant-associated microbiome is a critical frontier in agronomic research, with microbes interfacing with plants directly as both pathogens and symbionts. Microbes are also significant in their role as providers of ecosystem services, driving the cycling of nutrients and effecting the function of agrichemicals. Studying these populations and their interactions may lead to significant improvements in agricultural and environmental management techniques. The unique cropping system found in lowbush blueberries, in which wild plant populations are managed through environmental interventions, provides a valuable opportunity to study plant microbiome interactions in an agroecological context. Microbiome approaches can help to determine microbiome responses to agricultural inputs and management. When combined with other forms of analysis, such as microscopy, plant tissue content, and soil chemistry, microbiome sequencing can illuminate microbial functions, their interactions with one another, and the effect that they have on plant health. This suite of techniques thus offers the possibility to predict the effects of agricultural management on both ecological processes and crop outcomes on a granular level. Implementing these techniques in the context of lowbush blueberries not only has the potential improve blueberry management practices, but also may potentially lead to the development of similar techniques and understandings in other crops.

CHAPTER 3

3.1 Introduction

Microbe-plant interactions play a critical role in the agroecological system, and understanding these relationships is a significant frontier in plant ecosystems management. These symbiotic relationships are the product of concurrent evolution between plants and microbes and are, in many cases, necessary to ensure that the plant will thrive (Zilber-Rosenberg and Rosenberg 2008). Microbe-plant interactions may be particularly important in case of lowbush blueberries (a heterogeneous population consisting of *Vaccinium angustifolium* and *Vaccinium myrtilloides*) which are grown as a managed wild crop throughout the Canadian Atlantic Provinces, Quebec, and Maine. With over 67,000 hectares devoted to their production, lowbush blueberries are the most widely produced fruit crop in Canada by area of production (Agriculture and Agri-Food Canada 2019).

Lowbush blueberries occur naturally on sandy acidic soils. Commercial production consists of managing pre-existing, wild populations and using practices including mechanical pruning and integrated pest management (Drummond 2012; Esau et al. 2015, 2019). As lowbush blueberries are grown in their native habitat, spawned directly from wild populations, it stands to reason that managed lowbush blueberries may exhibit a particularly intimate relationship with the soil microbiome. Additionally, plants in the genus *Vaccinium*, as with many members of order Ericales, develop a distinctive mycorrhizal relationship with fungal species from the phyla Ascomycota and Basidiomycota known as ericoid mycorrhizae (Smith and Read 2008; Brundrett and

Tedersoo 2018; Yang et al. 2018a). These fungi form coils of hyphae within the host root cell, with each cell being individually colonized from the root surface (Brundrett and Tedersoo 2018).

The crop is produced using a two-year cycle. After harvest, the field is mechanically pruned close to ground level and the crop spends the next year in a period of formation of new shoot uprights with floral induction and initiation occurring in mid-summer [9]. The resulting floral bud growth and development occurring through to late autumn (Yarborough et al. 2017). The following year, bloom occurs with floral densities in excess of 350 million flowers per hectare needing to be pollinated and fertilized (Percival 2013). The berries are typically harvested 70 days after anthesis, after which the management cycle repeats. Lowbush blueberry crops are managed with a number of fungicidal compounds in order to maintain a healthy canopy free of leaf disease pressures including Septoria leaf spot, blueberry rust, and Valdensinia leaf spot. Left unabated, these leaf diseases can cause extensive damage to the canopy resulting in premature defoliation, inadequate carbohydrate supply for plant growth and development, and significantly reduced berry yields (Percival and Dawson 2009). While the effects of these chemical treatments on the plant itself have been well-studied, little is known about their effect on the plant microbiome. Given the interconnected nature of the blueberry and its native soil, combined with the potentially-disruptive effects that these fungicides may have on the lowbush blueberry microbiome, developing a rigorous understanding of the effects that fungicides have on agriculturally-relevant microbes and the microbiome is crucial in

advancing production techniques for this crop and in preserving this valuable natural resource.

The question of the effects of pesticides on the health of the soil microbiome is one of considerable environmental concern and has, as a result, been extensively studied. However, given the multiplicity of pesticides, both in terms of target organism (insecticide, fungicide, herbicide, etc.) and active ingredient mode of action, mobility, and persistence, it can be difficult to predict the effects of a given pest control product to a soil's ecosystem. For instance, fungicides have been linked to an increase in soil organic matter and, as a result, microbial activity (Pagano et al. 2017). Conversely, the effect of fungicides on the health of the soil microbiome tends to be deleterious. The application of many fungicides resulted in decreased levels of soil biological carbon and nitrogen, and had variable effects on the nitrogen cycle depending on the environmental context of the experiment (Ullah and Dijkstra 2019).

In order to further understanding of the ecotoxicology of fungicides in soil ecosystems, in general, and in the lowbush blueberry crop system, in particular, this study uses molecular genomics techniques to analyse the microbiomes of soils treated with two widely used fungicides, prothioconazole (Proline 480 SC) and chlorothalonil (Bravo 500) that differ in their mode of action and half-life in soil and are typically used to control leaf diseases. While work has been done on the subject of pesticide-microbiome interactions (Ullah and Dijkstra 2019), given the context-dependent effects of pesticides on the soil ecosystem, it is important to investigate the way that specific fungicides affect specific agroecosystems. Given the potentially disruptive fungicidal effects, this study was aimed

to evaluate the effect of prothioconazole and chlorothalonil, on the diversity, structure and function of soil microbiome. The choice of the fungicide was defined by their differences in mode of action, differences in uptake and mobility within plant tissue, and half-life in soil. Prothioconazole is an inhibitor of ergosterols has been demonstrated to have a short half-life of under 5.82 days (Parker et al. 2013; Lin et al. 2017), while chlorothalonil causes cell death by disrupting enzymatic action and has demonstrated to persist in the soil and to lead to reduced levels of soil respiration 60 days after application (Tillman et al. 1973; Wu et al. 2012). Additionally, chlorothalonil has been found to be more prone to run-off compared to prothioconazole (Deb et al. 2010). It was thus hypothesized that the two fungicides would induce significant changes to the fungal and bacterial microbiomes relative to control, and that the two fungicides would elicit significantly different effects from one another. Additionally, it was expected that the fungicide treatments may lead to changes in the function of the bacterial microbiome.

3.2 Materials and Methods

3.2.1 Site and Sampling

The soil samples were collected from a fungicide trial conducted in a commercial blueberry production field, in the first year of its crop cycle, in Highland Village, Nova Scotia (45.40406887, -63.6679066) on August 28th, 2019. Soil samples taken on June 1st 2021 revealed the soil at the sampling site to be highly acidic with a mean pH of 4.98. Additionally, the field was low in organic matter with a mean organic matter content of 3.95%. Visual observations of the soil suggested it to be of a sandy character, and outside of the topmost centimeter, the soil possessed a light orange color. This observation

conforms to the soil test results regarding organic matter and suggests a low retention rate of humic compounds. Soil surveys conducted by the Canadian government described the soil of the region as an orthic humo-ferric podzol and noted the soil of the region to be strongly acidic (Government of Canada n.d.). Soils such as these are typical of lowbush blueberry production, with sand and high acidity being common features of lowbush blueberry fields and acidity in particular has been associated with optimum plant growth in this context (Hall et al. 1964; Marty et al. 2019b). Samples were taken from two fungicide treatment groups (prothioconazole, and chlorothalonil), as well as a control treatment to which no fungicide was applied. For each of the three treatments, there were four replications with each replication consisting of a 4 x 6 m plot with a 2 m buffer strip separating each plot from the next. The fungicides had been applied to each of their respective plots three times prior to sampling, on July 4, July 15, and July 26, 2019. Prothioconazole was applied at a rate of 151 g a.i.·ha^{-1}, while chlorothalonil was applied at a rate of 3600 g a.i.·ha^{-1}. Both fungicides were applied using a Bellspray Inc. Model GS hand-held sprayer unit. Three samples of topsoil were taken from each plot for a total of 12 samples per treatment group. In total, 36 soil samples were acquired and kept on ice until they were returned to the laboratory. Upon arrival at the laboratory, each soil sample was sifted through a 2 mm sieve and then stored at -80 $^{\circ}$C until DNA extraction could be performed.

3.2.2 DNA Isolation and Sequencing

For each sample, 0.250 g (wet weight) of sifted soil was used for DNA extraction. Extraction was performed using the Omega Biotek E.Z.N.A. Soil DNA extraction kit (Omega

Bio-tek, Inc., Norcross, GA, United States) according to the manufacturer's specifications. Extracted DNA samples were subsequently stored at -20 ˚C. Prior to sequencing, the extracted DNA was qualified with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, United States) to determine the concentration of the genetic material. 5 µL of each extracted DNA sample was sent to the Dalhousie University CGEB-IMR for library preparation and sequencing with the Illumina MiSeq platform with paired-end 300+300 bp reads, in accordance with the PCR procedure, primers, and sequencing details outlined in the Microbiome Helper protocol (Comeau et al. 2017). The DNA was sequenced for the fungi-specific ITS2 region (ITS2: GTGAATCATCGAATCTTTGAA forward primer, TCCTCCGCTTATTGATATGC reverse primer) as well as prokaryotic V6-V8 16S rRNA (16S: ACGCGHNRAACCTTACC forward primer, ACGGGCRGTGWGTRCAA reverse primer) (Blaalid et al. 2013; Srinivasan et al. 2015).

3.2.3 Sequence Processing

The sequences were trimmed of their primers using QIIME2's Cutadept plug-in (Martin 2011; Comeau et al. 2017). The overlapping paired-end forward and reverse reads were stitched together using the QIIME2 VSEARCH wrapper (Rognes et al. 2016). Low-quality sequences were filtered from the dataset using QIIME2's q-score-joined function. Using QIIME2's Deblur plug-in, the sequences were organized into amplicon sequence variants (ASV), high resolution genomic groupings (Comeau et al. 2017; Amir et al. 2017; Callahan et al. 2017). In order to account for potential MiSeq bleed-through between runs (estimated by Illumina to be less than 0.1%), ASV which accounted for less than 0.1% of the total sequences were removed (Comeau et al. 2017). Taxonomic classifications were

assigned to the ASV using QIIME2's naïve-Bayes scikit-learn function, referencing SILVA databases (16S V6-V8, and fungi-specific ITS2) (Quast et al. 2013; Bokulich et al. 2018). ASV that had a high probability of being the product of chimeric reads were removed from the dataset by filtering out any ASV which were unassigned at the division of level. Additionally, ASV assigned to mitochondria and chloroplasts were filtered out (Comeau et al. 2017). These ASV were used to construct two tables of ASV counts per sample, one each for the 16S and ITS2 datasets, along with a listing of all taxa present, and a phylogenetic tree for both datasets (Comeau et al. 2017). A preliminary analysis of 16S rRNA data revealed one sample from UTG to have much higher concentrations of both the phyla Firmicutes and Bacteroidetes compared to the other samples. As these phyla are heavily associated with fecal material (Costea et al. 2017), it was determined that this sample had likely been contaminated with fecal matter and it was subsequently discarded from both the 16S and ITS2 datasets.

3.2.4 Data Analysis and Statistics

QIIME2's diversity function was used to calculate both Shannon and Simpson's indices (alpha diversity) as well as UniFrac matrices (beta diversity) for both datasets (Lozupone and Knight 2005; Kim et al. 2017). These UniFrac matrices were then subjected to an ADONIS test through which their values were fitted to a linear regression to determine what proportion of variance in community structure could be attributed to treatment. Principal Coordinates Analysis (PCoA) was performed using QIIME2 on the weighted UniFrac matrices. The UniFrac PCoA files were ported to RStudio using the qiime2R package and plotted using ggplot2 (Wickham 2016; Bisanz 2018). Differential relative

abundance analysis was performed with the STAMP software using Welch's t-test to identify taxa whose relative abundance varied significantly between treatments (Parks et al. 2014). Adjusted p-values were calculated using the Benjamini-Hochberg FDR multiple-test correction.

3.2.5 Functional Potential Analysis

Using the 16S rRNA based ASV tables and reference sequences generated by QIIME2, functional potentials of the bacterial community were predicted using the PICRUSt2 software. Through this method, relative abundance tables were generated both for complete MetaCyc functional pathways as well as individual enzymes, categorized by Enzyme Commission (EC) numbers (Ye and Doak 2009; Louca and Doebeli 2018; Barbera et al. 2019; Czech et al. 2020; Douglas et al. 2020). The relative abundances of these pathways and enzymes were tested for significantly differential relative abundance between treatment and group using the ALDEx2 software (Fernandes et al. 2013). This data was then graphically plotted using the ggplot2 package in R (Wickham 2016).

In addition to being categorized by their specific treatment (prothioconazole, chlorothalonil, and untreated control (UTG)) and compared to each other (Treatment), the samples were further categorized into groups based on whether or not they had received a fungicide treatment. Both prothioconazole and chlorothalonil treatments were thus aggregated into a combined treatment group (CTG) to be compared to UTG (Group).

3.3 Results

3.3.1 Data Description

Analysis of 16S rRNA data revealed one sample from UTG to have much higher concentrations of both the phyla Firmicutes and Bacteroidetes compared to the other samples. As these phyla are heavily associated with fecal material, it was suspected that this sample had been contaminated with fecal matter and it was subsequently discarded from both the 16S and ITS2 datasets (Carruthers et al. 2019). From the ITS2 dataset, two samples, both taken from the chlorothalonil treatment group, were deemed failed, as they contained fewer than 800 reads (416 reads and 140 reads, respectively). Those samples were discarded. From the 16S dataset, all non-contaminated samples were included. After the QIIME2 filtration processes had been performed, the two failed samples had been removed, the ITS2 dataset contained a total of 220,326 reads spread across 33 samples, with a mean per-sample frequency of 6,677 reads/sample and a median frequency of 4137 reads/samples. For normalization purposes, the ITS samples were rarefied to a depth of 874 reads/sample, for a total of 28,842 reads. The 16S dataset consisted of a total of 835,577 reads across 35 samples, with a mean frequency of 23,874 reads/sample and a median frequency of 24,733 reads/samples. The 16S samples were normalized to a depth of 2400 reads/sample, for a total of 84,000 reads evaluated.

3.3.2. Overall community composition

Basidiomycota was the major fungal division identified in our study and was represented by 88% of the total reads, Mortierellomycetes was the second most relatively

abundant division represented by 7% of the total ITS2 reads, with the remaining 5% of reads being from the remaining divisions. Within Basidiomycota, Clavariaceae was the most relatively abundant family, comprising 60% of the total reads, followed by Tricholomataceae, Hydnodontaceae and Serendipitaceae (4% of the total ITS2 reads each) (Fig. 1). Mortierellaceae was the most abundant Mortierellomycete found in the microbiome (7% of the total ITS2 reads).

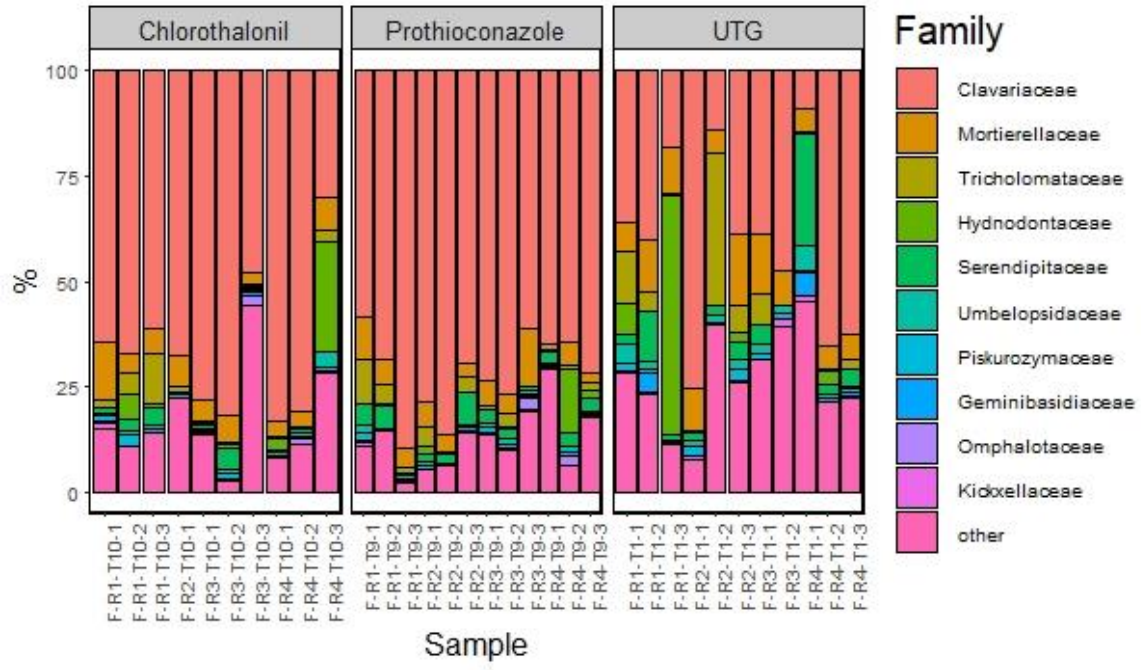

Figure 1: Relative abundances of fungal families identified in soil samples, by treatment group.

The five most relatively abundant bacterial phyla, which, combined, comprised 95% of the total bacterial community, were Proteobacteria (43%), Acidobacteria (24%), Actinobacteria (19%), Verrucomicrobia (7%), and Bacteroidetes (3%) (Fig. 2). At the class level, the five most relatively abundant taxa were found to be Alphaproteobacteria (30%),

Acidobacteria (20%), Actinobacteria (8%), Thermoleophilia (8%), Gammaproteobacteria (7%), in total comprising 74% of the total taxa present.

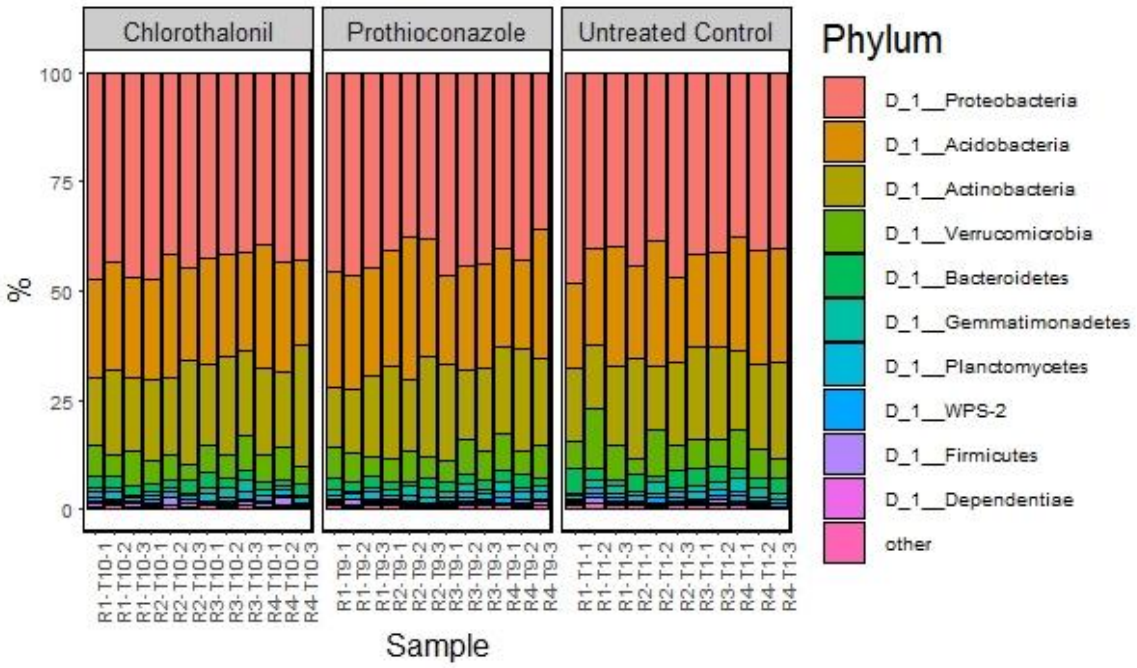

Figure 2: Relative abundances of bacterial phyla identified in soil samples, by treatment group.

3.3.3. Effect of fungicides application on microbial local diversity

The Shannon diversity indices of fungal CTG was found to be significantly lower than those of UTG ($p < 0.05$) (Table 1). The Shannon diversity indices of the prothioconazole treated fungal community, when tested independently, was also significantly lower than those of UTG ($p < 0.05$), while the Shannon diversity indices of chlorothalonil treated fungal community was found not to differ from UTG ($p > 0.05$). Compared to UTG, CTG fungal microbiome had significantly lowered Simpson's evenness ($p < 0.05$). However, neither fungicide, when compared independently to UTG, was found to be significantly different in its effect on fungal evenness ($p > 0.05$) and Simpson's index variance between

prothioconazole and chlorothalonil was not found to be significant ($p > 0.05$) (Table 1). Pairwise comparisons of Simpson's evenness, and Shannon diversity indices did not reveal significant differences between CTG and UTG in bacterial alpha-diversity, as well as between prothioconazole and UTG, chlorothalonil and UTG, and between prothioconazole and chlorothalonil treated soils.

Table 1. Shannon richness and Simpson evenness of fungal and bacterial communities.

Category	Shannon	Simpson	Shannon	Simpson
	ITS2		16S rRNA	
UTG	3.769^{A}	0.786^{A}	9.217^{A}	0.996^{A}
Prothioconazole	3.158^{B}	0.791^{AB}	9.133^{A}	0.996^{A}
Chlorothalonil	3.098^{AB}	0.789^{AB}	9.012^{A}	0.995^{A}
CTG	3.159^{B}	0.790^{B}	9.072^{A}	0.996^{A}

[1] Significance of variance tested by Kruskal-Wallis test of alpha-diversity indexes by Treatment and Group. For each variable, data followed by different letters are significantly different ($p < 0.05$).

3.3.4 Effect of fungicides application on communities dispersion

Visualization of dissimilarity between fungal communities across treatments (prothioconazole vs. chlorothalonil vs. UTG) revealed limited clustering or visible trends in beta diversity (Fig. S1.A). The analysis of strength and statistical significance of sample groupings (ADONIS test) indicated that the treatments influenced the structure of the fungal community ($R^2 = 0.11$, $p < 0.05$; Table 2). Additionally, comparing CTG to UTG

indicated that 10% of fungal community variance can be attributed to whether a field was treated with fungicide (R^2 = 0.099, $p < 0.01$). Conversely, the type of fungicide (prothioconazole vs. chlorothalonil) was not a driving factor affecting fungal community structure ($p > 0.1$; Table 2). The fungicide treatments imparted a lesser effect on the bacterial community structure compared to the fungal microbiome. There was no visual separation between the treatment groups based on weighted UniFrac distances (Fig. S1.B), as well as no statistical significance of sample groupings into CTG and UTG, treatments, or prothioconazole and chlorothalonil was detected (Fig. S1.B; Table 2). However, the structure of bacterial communities treated with chlorothalonil differed significantly from UTG ($p > 0.05$), around 9% of community variation was explained by chlorothalonil treatment.

Table 2. Variation in amplicon sequencing sample groupings explained by weighted UniFrac dissimilarity.

Grouping	ITS2	16S rRNA
Treatment	0.113*	0.073
Group (CTG vs. UTG)	0.099**	0.051
Prothioconazole vs. Chlorothalonil	0.024	0.025
Prothioconazole vs. UTG	0.139**	0.063
Chlorothalonil vs. UTG	0.101*	0.092*

[1] Weighted UniFrac distances were calculated for each subset of samples. ADONIS tests were used to assess whether beta-diversity is related to sample groupings, 999 permutations, R^2, **p < 0.01 and *P<0.05.

3.3.5. Effect of fungicides application on soil community structure

Fungal family, Clavariaceae, was found to have an increased relative abundance in plots treated with prothioconazole compared to UTG ($p < 0.05$). Interestingly, this difference in relative abundance was not present in comparisons of chlorothalonil and UTG. The relative abundance of a member of Clavariaceae, *Clavaria sphagnicola*, was also increased in the prothioconazole treatment group ($p < 0.05$) compared to UTG (Fig. S2). In comparing chlorothalonil and UTG, no taxa at any level of classification were found to differ significantly in their relative abundances ($p > 0.05$). Furthermore, no taxa were differentially represented between prothioconazole and chlorothalonil treatment groups ($p > 0.05$). In the 16S rRNA dataset, one bacterial genus, *Rudaea*, was found to be

significantly more relatively abundant in UTG compared to CTG ($p < 0.05$). However, no taxa were found to differ significantly in relative abundance between the soils treated with either fungicide individually and UTG, nor did any taxa differ between prothioconazole and chlorothalonil treated soils.

3.3.6. Effect of fungicides application on bacterial functional potentials

Based on 16S rRNA sequencing data, in total 2096 EC comprising 396 MetaCyc pathways were identified in the study. An analysis of the strength and statistical significance of sample function groupings (ADONIS test) indicated that individual treatments (prothioconazole vs. chlorothalonil vs. UTG) were not associated with differences in bacterial microbiome's functional composition considering both individual enzymes (EC) and functional pathway (p-value > 0.05) (Table 3). However, when comparing pathway relative abundances by group (CTG vs. UTG), a small but statistically significant functional variations between CTG and UTG ($R^2 = 0.056$, p-value < 0.05) was detected.

Table 3. Variation in functional pathway and EC sample groupings explained by weighted Bray-Curtis dissimilarity.

Grouping	Pathway	EC
Treatment	0.078	0.088
Group (CTG vs. UTG)	0.056*	0.068
Prothioconazole vs. Chlorothalonil	0.036	0.032

Grouping	Pathway	EC
Prothioconazole vs. UTG	0.057	0.068
Chlorothalonil vs. UTG	0.083	0.101

[1] PICRUSt2 pathway and CE tables were used with QIIME2 pipeline for ADONIS tests to assess whether their Bray-Curtis dissimilarity were related to sample grouping, 999 permutations, R^2, *P<0.05.

Four predicted biological pathways were differentially represented between UTG to CTG ($p < 0.1$). PWY-5676 (acetyl-CoA fermentation to butanoate II), PWY-6588 (pyruvate fermentation to acetone), and PWY-6641 (superpathway of sulfolactate degradation) were significantly increased in their relative abundances in CTG relative to UTG. One, PWY-7003 (glycerol degradation to butanol), was overrepresented in UTG relative to CTG (**Figure 3**). PWY-6641 was also overrepresented in plots treated with chlorothalonil treated soils compared to UTG, while no pathways were found to differ significantly between prothioconazole treated soils and UTG.

In total 109 ECs were differentially represented between CTG and UTG. The 71 highly-abundant ECs are listed in (Table S2). Together they comprised around 3.5% of total predicted feature counts from 16S rRNA reads. A highly abundant enzyme, nitronate monooxygenase, EC:1.13.12.16 was significantly increased in CTG relative to UTG. Another potentially consequential enzyme to the processing of nitrogen in the soil is EC:1.17.1.4 (xanthine dehydrogenase). Additionally, two enzymes, EC:3.8.1.2 and EC:3.8.1.3 ((S)-2-haloacid dehalogenase and Haloacetate dehalogenase respectively) were found to have significantly increased in relative abundance in CTG relative to UTG.

25 EC involved in xenobiotics biodegradation or metabolism were overrepresented in fungicide treated soil (Fig. 4), including highly abundant ECs – glutathione transferase (EC:2.5.1.18), isoquinoline 1-oxidoreductase (EC:1.3.99.16), 4-carboxymuconolactone decarboxylase (EC:4.1.1.44), gluconolactonase (EC:3.1.1.17), hippurate hydrolase (EC:3.5.1.32), and 3-oxoadipate enol-lactonase (EC:3.1.1.24). They represented around 43% of predicted feature counts comprising all ECs differentially represented between CTC and UTG.

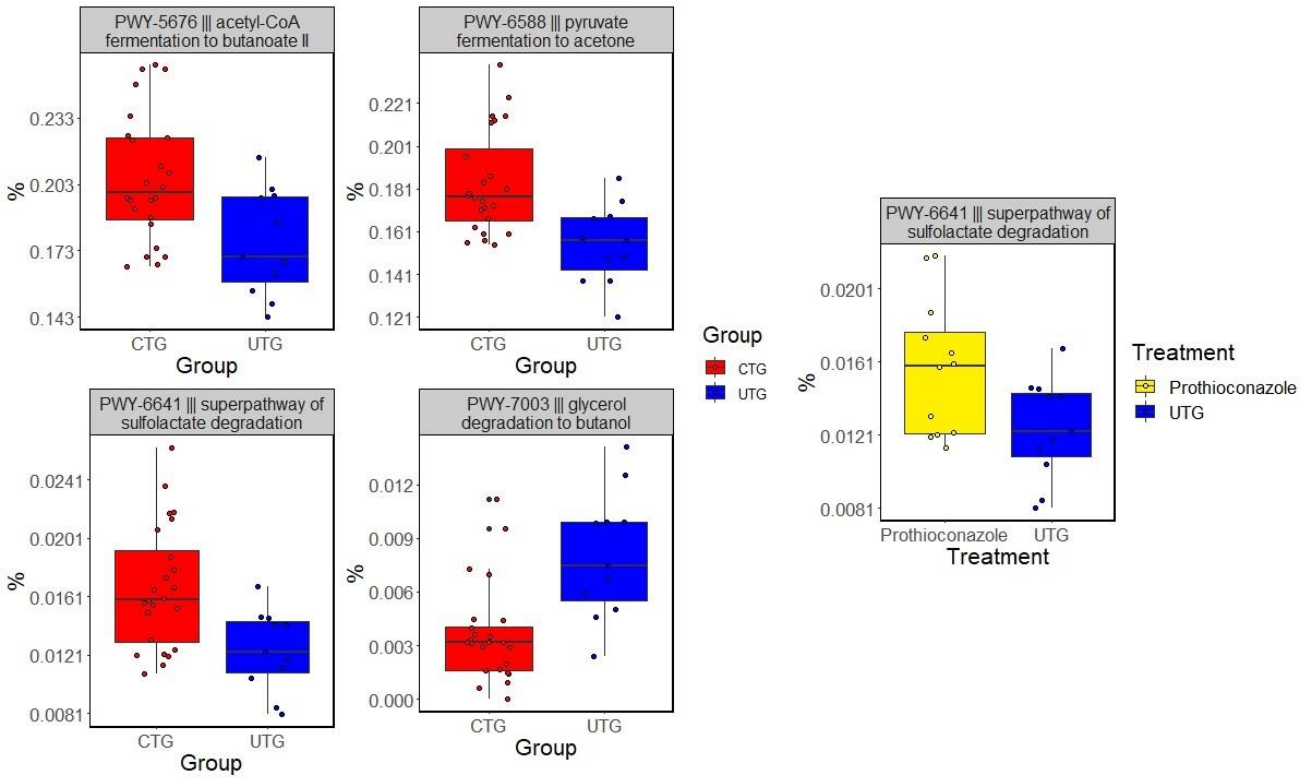

Figure 3. Pathways that were at differential relative abundances between CTG and UTG. Corrected p-values (q-values) were calculated based on Benjamini–Hochberg FDR multiple test correction. Features with (Welch's t-test) q-value < 0.1 in ALDEx2

were considered significant and were thus retained. The analysis was based on 16S rRNA sequencing data.

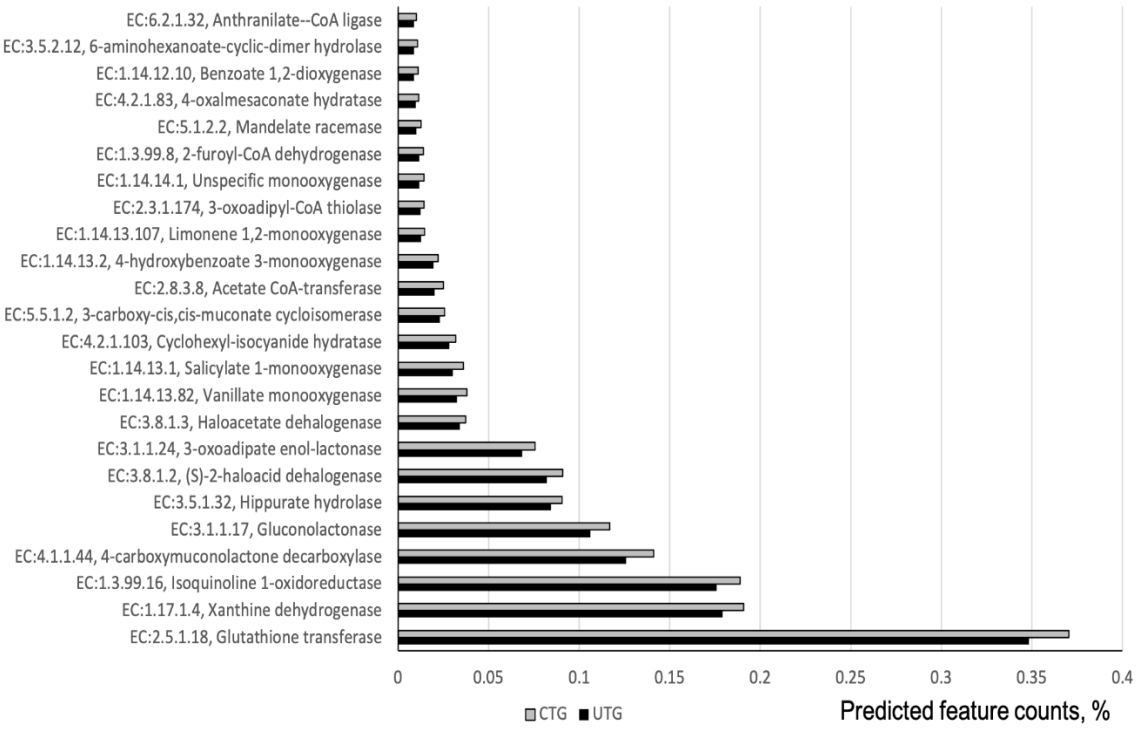

Figure 4. ECs involved in synthetic chemical degradation that were at differential relative abundances between CTG and UTG. Corrected p-values (q-values) were calculated based on Benjamini–Hochberg FDR multiple test correction. Features with (Welch's t-test) q-value < 0.1 in ALDEx2 were considered significant and were thus retained. The analysis was based on 16S rRNA sequencing data.

4. Discussion

Our data indicated that application of the fungicides prothioconazole and chlorothalonil had some effect on soil microbiome, but bacterial and fungal communities differed in their responses to the treatments, as reflected in the changes in the

communities' structure and/or diversity. When combined in one group (CTG), the fungal microbiomes from fungicide treated soils exhibited decrease in alpha-diversity and fungicide treatment was a driving factor affecting fungal community structure. However, no response of bacterial microbiome to fungicides treatments was detected. Interestingly, when compared to each other, despite their differing mechanisms of action and degrees of persistence in the soil, there was no significant difference between the two fungicides based on their overall effect on community structure (ADONIS test) and alpha diversity.

Considering individual fungicides, the treatment with chlorothalonil significantly affected both fungal and bacterial communities' structure. Previous research on the effects of chlorothalonil application on bacterial communities have had mixed findings. In a 2019 study, it was found that the growth of 33% of the strains, isolated from the soils exposed to chlorothalonil, was affected by the presence the fungicide (Díaz Rodríguez et al. 2019). However, other trials have identified an increased growth in some bacterial taxa in the presence of chlorothalonil (Baćmaga et al. 2018). The effect of chlorothalonil on the soil microbiome was not surprising as chlorothalonil is persistent in the soil and has been shown to reduce soil respiration (Wu et al. 2012; Baćmaga et al. 2018). It was perhaps somewhat more surprising that prothioconazole, with its relatively shorter half-life, led to the loss in fungal richness as well as affected fungal community structure (ADONIS test). However, given that the fungicides are foliar treatments, in this context, it is perhaps not surprising that the two treatments did not yield more dramatic changes to soil microbial community structure relative to control as the amount of the fungicide-soil contact would not be expected to be large. An additional factor which may mitigate the effects of the

two treatments on soil communities may be the physical and chemical characteristics of the soil itself. Given the coarse texture of the soil, its capacity for water retention is relatively low, and its low pH minimizes amount of adsorption in the soil matrix. It may be the case that these two factors combine to minimize the amount of time that any fungicide remains in the soil further reducing its impact on the soil microbiome.

Clavariaceae was found to be the most relatively abundant fungal family in all types of soils. The relative abundance of *Clavariaceae,* and more specifically *Clavaria sphagnicola,* was increased in prothioconazole treated soils. *C. sphagnicola* may be of agricultural relevance to the lowbush blueberry as it had been putatively associated with the formation of ericoid mycorrhiza associations (EMA) with plants in the genus *Vaccinium* and may exist in a symbiotic relationship with the blueberry plant (Yang et al. 2018a). One possible explanation for the expansion of *C. sphagnicola* may be that it fills niches which have been thinned out by fungicide treatment. If indeed *C. sphagnicola* is forming EMA with the blueberry crop, the increased relative abundance of *C. sphagnicola* may imply that it is replacing other symbionts or pathogens which are killed by the fungicide treatment, possibly implying a limited loss of overall mycorrhizal symbiosis. Alternatively, it may be the case that the plants, with the burden of disease lessened, have greater photosynthetic resources to spare and can support a larger number of symbionts per plant.

Analysis of functional compositions of soil microbiome identified four pathways differentially represented between UTG and CTG. Pathways PWY-5676, PWY-6588 and PWY-7003 were associated with anaerobic respiratory pathways. PWY-5676 and PWY-

6588 were overrepresented in CTG, and PWY-7003 had an increased relative abundance in UTG. Superpathway of sulfolactate degradation, PWY-6641, was overrepresented in CTG compared to UTG. PWY-6641 is an umbrella category of three related pathways through which bacteria convert organosulfonate into sulfite and either pyruvate or acetyl-CoA. Additionally, PWY-6641 was found to have increased significantly in relative abundance in plots treated with chlorothalonil in comparison to UTG. The overrepresentation of this pathway may imply a change to the way in which sulfur compounds are processed in the soil induced by either chlorothalonil or fungicide treatment in general.

Variation in the relative abundances of a number of enzymes between UTG and CTG suggested changes in the function of bacterial microbiome under fungicide treatments. A highly abundant enzyme, nitronate monooxygenase, EC:1.13.12.16 was significantly increased in CTG relative to UTG. This enzyme is degrading aci-nitroethane into acetaldehyde converting a nitrogenous organic compound into an inorganic plant-available nitrogen compound ("ENZYME - 1.13.12.16 Nitronate monooxygenase" n.d.). Another potentially consequential enzyme to the processing of nitrogen in the soil is EC:1.17.1.4 (xanthine dehydrogenase), which processes xanthine into urate ("ENZYME - 1.17.1.4 Xanthine dehydrogenase" n.d.). The increase in the relative abundances of these enzyme under CTG may be an indicator of changes to the way in which the soil nutrients become available to the plants. Additionally, two enzymes, EC:3.8.1.2 and EC:3.8.1.3 ((S)-2-haloacid dehalogenase and haloacetate dehalogenase respectively) pertaining to the degradation of halo-organic compounds were found to have significantly increased in

relative abundance in CTG relative to UTG ("ENZYME - 3.8.1.2 (S)-2-haloacid dehalogenase" n.d.; "MetaCyc S-2-haloacid dehalogenase" n.d.). This finding is significant in that both prothioconazole and chlorothalonil are themselves halocarbons, potentially alluding to the breakdown of pesticides by microbial action. Several other enzymes involved in synthetic chemical degradation and metabolism were found overrepresented in fungicides treated soils. These findings suggested a functional shift toward degradation of synthetic chemicals explained by the introduction of the fungicides into soil. Additionally, this apparent increase in bacterial degradation of fungicides may suggest the possibility of a reduction in fungicide effectiveness in certain contexts. If bacteria reduce the time in which the active ingredient is present, the total time during which the plant is protected from disease pressure is reduced. Indeed, it has been observed that the microbial selection process induced by repeated applications of a given fungicide may lead to an accelerated rate of biodegradation (Arbeli and Fuentes 2007). The increased relative abundance of these enzymes may be associated with this phenomenon and would therefore imply that the efficacy of the trial fungicides would decrease over time.

Despite some differences in relative abundances of several enzymes and functional pathways between CTG and UTG, where was a minor effect of fungicide treatments on the overall bacterial functional composition. While the analysis of the strength and statistical significance of sample function groupings did find that a small proportion of functional variation in pathways between CTG and UTG, comparing the individual treatment groups did not return significant results.

Considering EC relative abundances, no significant variation was found in response to either group or individual treatment. Some of the significantly differentially abundant enzymes mapped to reactions of potential ecological significance, suggest changes to the soil ecology as a result of fungicide application. Furthermore, though some variance in functional pathway relative abundances corresponded to fungicide intervention, the amount of variance was small enough that it is unclear whether fundamental changes to the soil's ecosystem services would be expected as a result.

5. Conclusions

Given increased awareness of the importance of the soil microbiome on soil health and crop production, developing an understanding of the effects of agrichemicals on the soil microbiome is equally critical. While both fungicides evaluated in this study were shown to diminish soil fungal diversity, our findings suggest that the effects of prothioconazole may have a less deleterious effect on crop symbionts as shown by the increased relative abundance of a taxa of potential blueberry symbionts. Analysis of the bacterial microbiome did not indicate significant changes to the taxonomic profile but the predicted functions of the microbiome under treatment conditions relative to control did suggest the possibility of changes to soil nutrient processing and suggested the breakdown of fungicides by bacterial action. This accelerated biodegradation could prove problematic for disease management by reducing fungicide efficacy. However, the use of biological fungicides in rotation with synthetic fungicides presents promise in terms of mitigating this effect.

CHAPTER 4

4.1 Introduction

The significant findings of CHAPTER 3 suggested that the interactions between fungicides and the soil microbiome of lowbush blueberries merited additional investigation. In comparing prothioconazole and chlorothalonil to untreated control plots, it was observed that those plots treated with a fungicide had a significantly lower fungal species richness relative to untreated plots. Community structure was found to vary significantly between the three treatment groups, with 11% of the overall community structural variance being attributable to the fungicide treatment, or lack thereof (Lloyd et al. 2021). Additionally, one fungal family which potentially represented blueberry symbionts was determined to have significantly increased in its relative abundance in plots treated with prothioconazole relative to the untreated control. Finally, in examining predicted bacterial function, four pathways were determined to have changed in relative abundance relative to control, as well as 109 enzymes. Of these, one of the pathways and several enzymes were thought to have implications on nutrient processing in the soil. Furthermore, 24 enzymes associated with the degradation of either halocarbons or other synthetic chemicals had increased in relative abundance in plots treated with either of the two fungicides relative to control. That shift strongly suggested that the bacterial community was thus engaged in the biodegradation of pesticides (Lloyd et al. 2021).

The findings of that study, those associated with potential symbionts and microbiome function especially, may be consequential in terms of soil or plant health. With that

guiding principle, this follow up study was conceived to gather further information on the effects of fungicides on the soil microbiome to better chart a path towards ecologically-sound blueberry management. Using those microbiome technologies outlined in CHAPTER 3, this investigation has broadened the number of fungicides evaluated to establish a more complete image of the interactions between fungicides and soil ecology (Lloyd et al. 2021). Nine commercially available products comprising six active ingredients formed the core of this trial. Six of the fungicides contained a single active ingredient, while three contained two active ingredients. In total, four mechanisms of action were present in the trial fungicides. This array of trial products allows for assessment of a wider range of active ingredients, to broaden the knowledge base set forth in CHAPTER 3, and to explore some interactive effects of the two fungicides.

Another new path that has been explored here, but which was not present in CHAPTER 3, is the potential for either the fungicides or the resultant changes to the soil microbiome to alter expressed plant phenotypes and crop outcomes. To understand these effects, stem samples were taken from each plot at two stages of the growing season to evaluate variance in plant growth and development, disease incidence and severity, and expected crop outcomes. By examining differences in those factors, and integrating that data with microbiome analyses, it is expected that any changes to plant growth and crop outcomes may be linked back to either the fungicide or related changes to the microbiome from which they originate. One final difference between this study and that of CHAPTER 3 pertains to the two-year cropping cycle of the lowbush blueberry. While the previous study was conducted on a field in its vegetative growth or “sprout” year, this trial was

conducted on a field in its crop year. Since the two years have different sources of disease pressure, fungicide applications will differ accordingly. Thus, to attain a more complete image of the ecological effects of fungicide usage in the lowbush blueberry crop system, it is necessary to examine both the sprout and crop years.

Fundamentally, some of the questions asked by this trial are the same as those asked in CHAPTER 3. What effects will fungicide applications have on bacterial and fungal diversity and community structure? Will the relative abundances of specific bacterial and fungal taxa be significantly altered by fungicidal interventions? However, this time, answers to these questions will be sought for a much larger set of fungicides, and in the different growth context of the crop year. Furthermore, this study asks the question of whether the fungicides in question have any impact on plant outcomes, either as a result of their own action or as an effect of changes that they impart to the soil community. Finally, the results of CHAPTER 3 will be compared to those of this study in the interest of determining what, if any, impacts of fungicide application are consistent. Given the diversity of active ingredients and mechanisms of action, it was expected that each fungicide application would lead to significant changes to microbial diversity and community structure relative to both the control and to the other fungicides. In the same vein, it was anticipated that some microbial taxa and functions would exhibit significantly altered relative abundance as a result of one or more of the fungicide treatments relative to control, with fungi likely showing a greater sensitivity to treatment. Finally, plant outcomes were expected to show some significant difference between treatment groups in a way attributable to either the fungicide or the soil microbiome.

4.2 Background of the fungicides used in this study

The nine fungicides tested in this trial are all systemic fungicides applied with the intent of defending the crop against fungal infection, in this case, predominantly *Monilinia* and *Botrytis*. In total, these fungicides contain six individual active ingredients. Six of the fungicides are single active ingredients, while three are composed of combinations of active ingredients (see **Table 1**). Additionally, the trial fungicides are divided into four groups as defined by the Fungicide Resistance Action Committee (FRAC), pertaining to their mode of action.

4.2.1 Pyraclostrobin

Pyraclostrobin is an inhibitor of the mitochondrial cytochrome bc_1 complex inhibitor (FRAC Group 11). Previous findings on the persistence of pyraclostrobin in the soil have been inconsistent. One study in southern China found the soil half-life of this compound to be 9.89 days based on four banana field trials (Fu et al. 2016). Conversely, soil tests on peanut fields in the area of Beijing found the half-life of the compound to be 16.5 days (Zhang et al. 2012). This variance may imply that a warmer climate has the effect of causing the compound to degrade at a quicker rate. Pyraclostrobin's mobility in the soil is relatively low, with soil column leaching tests finding that, even under conditions simulating heavy rainfall, leaching of the fungicide below 10cm would be minimal (Reddy et al. 2013). Laboratory tests of pyraclostrobin on fluvo-aquic soil (homogenized samples treated and incubated) have shown decreased dehydrogenase activity in soils. Furthermore, these tests demonstrated that several taxa were significantly changed in

relative abundance, carrying with it the implication that pyraclostrobin may have potential to disrupt or stimulate the phosphorous cycle (Zhang et al. 2019). Another observed characteristic of this fungicide is that it has been demonstrated to lead to changes in plant metabolism, leading to increased biomass in cereal plants (Kanungo and Joshi 2014) Finally, pyraclostrobin, when used as a seed treatment (under the trade name Stamina), was not found to lead to any significant differences in levels of AM fungi colonization in comparison to control in trials on corn, additionally the phosphorus uptake of the crop was not significantly altered by treatment (Cameron et al. 2017).

4.2.2 Pydiflumetofen

Pydiflumetofen (FRAC Group 7), is a new addition to the blueberry sector, and is an inhibitor of succinate dehydrogenase in the citric acid cycle and electron transfer chain. The European Food Safety Authority has reported that it is extremely persistent, with a half-life of 8540 days. However, it also reported that the compound poses a low risk to soil microorganisms (Arena et al. 2019).

4.2.3 Fluxapyroxad

Fluxapyroxad, like pydiflumetofen, is an inhibitor of succinate dehydrogenase (FRAC group 7). This active ingredient is very persistent, with a reported half-life of 157.6 days in soil with high organic matter contents, and longer half lives in soils with less accumulated organic matter (Li et al. 2015). Additionally, degradation was found to occur more slowly in acidic soil than in neutral soils (Li et al. 2015). These same trials found that, in lab tests, soil fungi biomass was decreased and the bacteria to fungi ratio was increased

by treatment with pydiflumetofen (Li et al. 2015). The treatment also increased the ratio of Gram-negative to Gram-positive bacteria in samples after the first 15 days of the trial (Wu et al. 2015).

4.2.4 Pyrimethanil

Pyrimethanil, an inhibitor of methionine production (FRAC group 9) used in the control of Botrytis, has been found by one study to lead to a general increase in bacterial richness, however the effects were temporary and within eight weeks no difference in richness remained between treated groups and control. This same study found that significant differences in community structure resulted from interactions between pyrimethanil and rainfall rates (Ng et al. 2014). Soil column leaching tests have shown pyrimethanil to have a minor propensity towards leaching (Fenoll et al. 2010).

4.2.5 Fludioxonil

Fludioxonil, (FRAC Group 12) has as a mechanism of action the inhibition of a catalytic enzyme which breaks down methylglyoxal, which in turn leads to aldehydic stress and eventually cell death. Furthermore, it has been shown to inhibit genes involved in a two-component signal transduction system which is found in bacteria, a fact which may imply an inhibiting effect on bacterial communities as a whole (Yang et al. 2011). Where the question of soil persistence is concerned, a study of fungicide residues in vineyard soils found that the half-life of the fungicide in soil ranged from 6.0-12.1 days depending on location. The low end of that range being detected from soils taken in a subtropical monsoon climate, the higher of the two half-lives was taken from a continental monsoon

climate. These findings may imply that warmer temperatures are responsible for greater microbial activity and thus faster fungicide degradation (Zhang et al. 2015). Tests of the fungicides dissipation rate in biobeds (purification systems designed to use a biological substrate, such as agricultural wastes, to trap and dissipate pesticides before they should leach into waterways) found the half-life of the compound to be significantly longer at 115.5 days. Additionally, fludioxonil has been linked to significant differences in microbial community structures (Marinozzi et al. 2013). However, in lab-based leaching/sorption tests, it has been found to be relatively hydrophobic and not prone to leaching (Smalling et al. 2018).

4.2.6 Fluopyram

Fluopyram (FRAC Group 7), is another inhibitor of succinate dehydrogenase. A study of fluopyram persistence on bell peppers found the compound to have a half-life of 29.7-31 days in the open field and a slightly longer half-life of 38.4-40 days in the greenhouse where the incoming sunlight is less intense. This discrepancy implies that light is a critical part of the degradation of the compound (Matadha et al. 2020). Contrasting these findings, Zhang et al. found the half-life of Fluopyram to be 64.2 days (Zhang et al. 2014b). This compound has been found to increase the number of P-solubilizing rhizosphere bacteria on pepper plants (Sun et al. 2020). Furthermore it has been found to increase the presence of N-fixing rhizosphere genes, and to not significantly alter the community structure or diversity of rhizosphere bacteria (Sun et al. 2020). Using a PCA of BIOLOG data to analyze carbon source utilization has shown that soils treated with fluopyram were distinct from those left untreated, suggesting significant differences in microbiome

function under fluopyram treatment (Zhang et al. 2014b). In that same study, they determined that soil fungi were significantly inhibited at all three dosages relative to control, while soil bacteria (both Gram positive and Gram negative) were also inhibited by all three levels of treatment (Zhang et al. 2014b).

4.3 Methods and Materials

4.3.1 Site and management

The fungicide trial was conducted at a commercial blueberry production field in Debert, Nova Scotia (45.438321, -63.453072) which was in the harvest year of the cropping cycle. As in CHAPTER 3, the soil of the region was an orthic humo-ferric podzol and has been noted in surveys to be highly acidic (Government of Canada n.d.). Each plot consisted of a 4m x 4m area separated from its neighbors by a 2m buffer zone. The experimental design consisted of nine fungicide treatments (Table 4), and one untreated control (UTG) across five replications, arranged in a randomized complete block design. Fungicides were applied as described in CHAPTER 3.

Table 4: List of fungicides used in trial

Trade Name	Active Ingredients	Application rate	Mode of Action
Cabrio	pyraclostrobin	1.95 g/ 100 m²	inhibits mitochondrial cytochrome bc-1 complex
Miravis Bold	pydiflumetofen	0.84 mL/100 m²	inhibits succinate dehydrogenase
Sercadis	fluxapyroxad	1.30 mL/100 m²	inhibits succinate dehydrogenase
Scala	pyrimethanil	11.25 mL/100m²	inhibits methionine production

Trade Name	Active Ingredients	Application rate	Mode of Action
Scholar	fludioxonil	2.04 mL/100 m²	inhibits a catalytic enzyme that breaks down methylglyoxal
Velum Prime	fluopyram	3.0 mL/100 m²	inhibits succinate dehydrogenase
Luna Tranquility	fluopyram + pyrimethanil	12 mL/100 m²	inhibits succinate dehydrogenase + inhibits methionine production
Miravis Prime	pydiflumetofen + fludioxonil	8.77 mL/ 100 m²	inhibits a catalytic enzyme that breaks down methylglyoxal + inhibits succinate dehydrogenase
Merivon	fluxapyroxad + pyraclostrobin	7.31 mL/100 m²	inhibits succinate dehydrogenase + inhibits mitochondrial cytochrome bc-1 complex

4.3.2 Stem sampling

From each plot in reps 1 through 4, fifteen stems were collected on July 8th. The length of each stem was measured, and the number of both floral and vegetative nodes was counted. Additionally, the vegetative and floral development stage of each stem was noted. Furthermore, the incidence and severity of *Monilinia* and *Botrytis* on both vegetative and floral nodes was taken into account. Finally, the presence of *Septoria*, was taken into account for each stem.

In August, shortly before harvest, 15 stems samples were taken from each plot. For each stem, the number of ripe berries was counted, as well as the number of berries which were underdeveloped or otherwise unmarketable. As with the first round of sampling, the length of each stem was taken into account as well.

4.3.3 Stem Analyses

To determine whether variance in each of these factors was significantly correlated to each fungicide treatment, an ANOVA test was conducted, followed by Fisher's LSD test to determine which treatments yielded results which differed significantly from each other (R Core Team 2019). These analyses were conducted for both rounds of stem sampling.

4.3.4 Soil sampling, DNA isolation, and Sequencing

From each plot, three soil samples were taken at random from the top 10 cm of soil, leading to a total of 150 soil samples. Soil samples were immediately placed on ice in the field and were sifted through a 2mm sieve before being transferred into a -80C freezer. DNA extraction was performed as described in CHAPTER 3. Initial DNA sequencing was performed as described in CHAPTER 3.

4.3.5 Sequence processing

Soil DNA samples were processed using QIIME2, following the procedure outlined in CHAPTER 3, with one exception. In this analysis, reads were joined using PEAR, rather than VSEARCH as was performed in the previous study (Zhang et al. 2014a). This substitution was made as PEAR demonstrated superior read retention for the ITS2 data in this trial.

4.3.6 Data analysis

As in CHAPTER 3, alpha diversity metrics were calculated and compared using QIIME2. UniFrac matrices were also generated using QIIME2, and their variance was compared

using the ADONIS test. Differential relative abundances for both 16S and ITS2 were determined using ALDEx2. Functional potentials were predicted and analysed as described in CHAPTER 3. All graphics were produced using ggplot2 (Wickham 2016).

4.4 Results

4.4.1 Phenotype expression, crop outcomes, and disease incidence

The average stem length at approximate harvest time was 16.03cm. Stem length was found to differ significantly as a result of treatment group ($p < 0.05$). Ten pairwise comparisons of treatments differed significantly from one another in terms of stem length, with six out of the ten significant pairings involving Scala, which was the only treatment whose stem length differed significantly from UTG **(Table 5**). However, the number of ripe berries, as well as the number of unmarketable berries per stem, did not vary significantly as a result of either fungicide treatment or mechanism of action. Additionally, stem lengths did not vary significantly with mechanism of action.

Table 5: Significantly different stem lengths by treatment (Fisher's LSD)

Trt1	Trt2	diff	Pval	Variable
Scala	UTG	-2.184	0.026	Stem Length
Scala	Cabrio	-2.305	0.019	Stem Length
Sercadis	Miravis Bold	2.499	0.011	Stem Length
Velum Prime	Miravis Bold	2.117	0.032	Stem Length
Scala	Sercadis	-3.518	0.000	Stem Length
Miravis Prime	Sercadis	-2.770	0.005	Stem Length
Velum Prime	Scala	3.136	0.001	Stem Length
Luna Tranquility	Scala	2.107	0.031	Stem Length
Merivon	Scala	2.095	0.032	Stem Length
Miravis Prime	Velum Prime	-2.389	0.015	Stem Length

Fungicide treatments had a significant effect on the rate of floral bud development ($p < 0.05$), with Miravis Prime having the least developed floral buds at sample time, and Merivon having the greatest degree of development at that time (**Table 6**). However, the number of floral nodes, as well as the number of vegetative nodes did not differ significantly with treatment. The prevalence and severity of *Monilinia* on vegetative nodes did not vary significantly between treatment groups ($p > 0.05$) and the pathogen was not observed on any of the floral nodes sampled. *Botryris* was found on the floral nodes of only one of the 539 stem samples analysed and was not observed on any of the vegetative nodes.

Table 6: Differing floral bud development stages by treatment

Treatment1	Treatment2	Mean difference (cm)	pval
Cabrio	UTG	-0.357	0.029
Scala	UTG	-0.357	0.026
Miravis Prime	UTG	-0.424	0.007
Miravis Bold	Cabrio	0.328	0.043
Scholar	Cabrio	0.393	0.016
Velum Prime	Cabrio	0.321	0.049
Merivon	Cabrio	0.464	0.005
Scala	Miravis Bold	-0.328	0.039
Miravis Prime	Miravis Bold	-0.394	0.012
Miravis Prime	Sercadis	-0.360	0.021
Scholar	Scala	0.393	0.014
Velum Prime	Scala	0.321	0.045
Merivon	Scala	0.464	0.004
Miravis Prime	Scholar	-0.460	0.004
Miravis Prime	Velum Prime	-0.388	0.014
Merivon	Miravis Prime	0.531	0.001

4.4.2 Data Characteristics

The fungal dataset, once contaminant sequences were removed, contained a total of 2,018 features, with a mean frequency of 15,753 reads/sample, with a total of 153 samples. Samples with a depth below 4,278 reads were excluded from analysis. As a result, 24 samples were excluded, bringing the final sample total to 129. This exclusion lowered the total number of identified features to 1,994 and raised the mean reads/sample to 18,251. For alpha and beta diversity analyses, the dataset was rarefied to the depth of 4,278 reads. However, for the comparison of relative abundances of both taxa and functional pathways, an unrarefied dataset was used for ALDEx2. Ascomycota and Basidiomycota were, by a long margin the most well-represented fungal phyla, together comprising more than approximately 75% of the reads in each sample.

Once contaminants were removed from the bacterial dataset, 9,635 features were present across 153 samples, with a mean reads/sample of 18,949 reads/sample. An acceptable minimum read count was found to be 5,089 reads/sample, with all samples below that threshold being removed from the dataset. Thus, 20 samples were excluded, reducing the total number of features to 9,631, and increasing the mean reads/sample to 21,249. Sample rarefaction was performed as described for ITS2. The majority of all reads corresponded to Proteobacteria, Actinobacteria, and Acidobacteria.

4.4.3 Alpha Diversity

4.4.3.1 Bacterial diversity

In the bacterial dataset, it was found that species richness, as measured using the Chao1 index, did not vary significantly as a result of treatment groups on the whole. Furthermore, a pairwise comparison between each treatment group did not find a significant difference in species richness between any two treatments. However, it did show that there were significant differences in species richness between UTG and CTG, with CTG exhibiting a higher species richness than UTG ($p < 0.050$) (**Figure 5**). In a similar fashion, species evenness, quantified with Simpson's evenness index, did not vary significantly as a result of individual treatment groups, either in terms of the dataset as a whole or in terms of pairwise comparisons. However, it was found that species evenness was significantly lower in CTG relative to UTG ($p < 0.05$) (**Figure 5**).

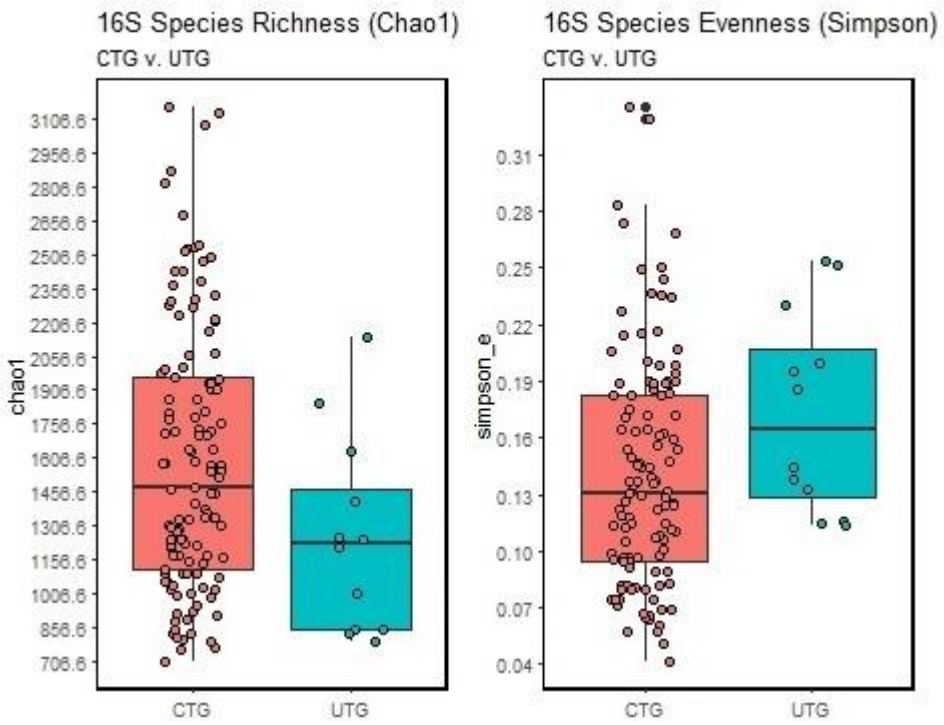

Figure 5: Alpha diversity metrics comparing bacterial populations of CTG and UTG. Species richness, as determined by the Chao1 metric was shown to be significantly higher in CTG relative to UTG. While Species Evenness, determined by Simpon's Evenness metric was reduced in CTG.

4.4.3.2 Fungal diversity

In contrast with the bacterial dataset results, no significant variance in species richness could be attributed to fungicide application, either in comparing individual treatment groups or in comparing CTG to UTG. Comparisons of species evenness also did not show any significant difference between either treatment groups or between CTG and UTG (**Figure 6**).

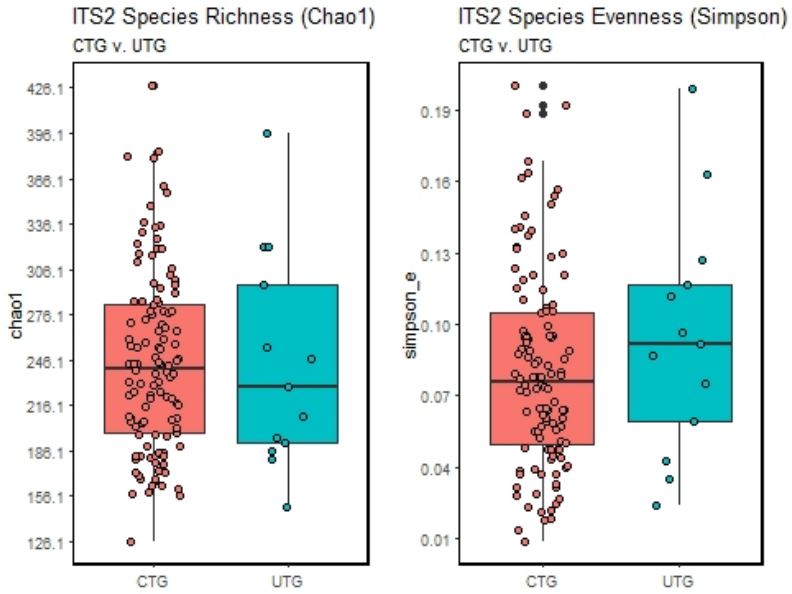

Figure 6: Alpha diversity for fungal population of both CTG and UTG. Neither richness nor evenness differed significantly between UTG and CTG.

4.4.4 Beta Diversity

4.4.4.1 Bacterial community

ADONIS tests comparing the significance of difference between UniFrac matrices for each treatment group found that 10% of variance in the 16S dataset could be attributed to the effect of individual treatments ($p < 0.05$). Additionally, pairwise ADONIS comparisons between individual treatment groups demonstrated that 9 treatment combinations had a significant difference in UniFrac matrices which could be attributed to treatment groups ($p < 0.05$). One treatment group Luna Tranquility, generated the greatest number of significant variances with other groups, comprising 6 of the 9 significant pairings (**Table 7**). Notably, Luna Tranquility was the only treatment group responsible for significant variance from control, causing 9% of the overall variance

between the two groups (Figure 7). In addition to the variance generated by Luna Tranquility, three other pairings presented significant differences as a result of treatment. Miravis Prime differed significantly from both Velum Prime and Sercadis (responsible for 13% and 9% variance respectively). Finally, Velum Prime differed significantly from Cabrio, generating 9% of the overall UniFrac variance.

Table 7: ADONIS test of 16S UniFrac Variance

Group 1	Group2	R2	p
Luna Tranquility	UTG	0.08	0.02
Luna Tranquility	Miravis Bold	0.08	0.023
Luna Tranquility	Sercadis	0.09	0.01
Luna Tranquility	Scholar	0.12	0.003
Luna Tranquility	Miravis Prime	0.11	0.007
Luna Tranquility	Merivon	0.08	0.029
Velum Prime	Cabrio	0.09	0.049
Velum Prime	Miravis Prime	0.13	0.002
Sercadis	Miravis Prime	0.09	0.018
All Treatments		0.10	0.009

4.4.4.2 Fungal community

With the fungal ITS2 dataset, Luna Tranquility was also the only treatment group which differed significantly different from control (9% of variance, $p < 0.05$) (**Figure 7**).

While Luna Tranquility, a fungicide with two active ingredients (fluopyram and pyrimethanil) elicited significant differences relative to UTG in both the fungal and bacterial datasets, neither Velum Prime (a.i. fluopyram), nor Scala (a.i. pyrimethanil) caused significant changes relative to UTG. In fact, in the fungal dataset, the largest difference in community structures was found between Luna Tranquility and Scala, with treatment being found accountable for 15% of variance. For both the 16S rRNA and ITS2 datasets, no significant differences were found in the community structure of CTG and UTG.

Table 8: ADONIS test of ITS2 UniFrac Variance

Group 1	**Group 2**	**R2**	**p**
Luna Tranquility	UTG	0.09	0.039
Luna Tranquility	Cabrio	0.09	0.036
Luna Tranquility	Miravis Bold	0.17	0.009
Luna Tranquility	Scala	0.15	0.002
Group 1	**Group 2**	**R2**	**p**
Luna Tranquility	Scholar	0.08	0.042
Luna Tranquility	Velum Prime	0.09	0.026
Luna Tranquility	Miravis Prime	0.13	0.008
Miravis Bold	Scholar	0.12	0.044
Miravis Bold	Merivon	0.11	0.046
All Treatments		0.01	0.163

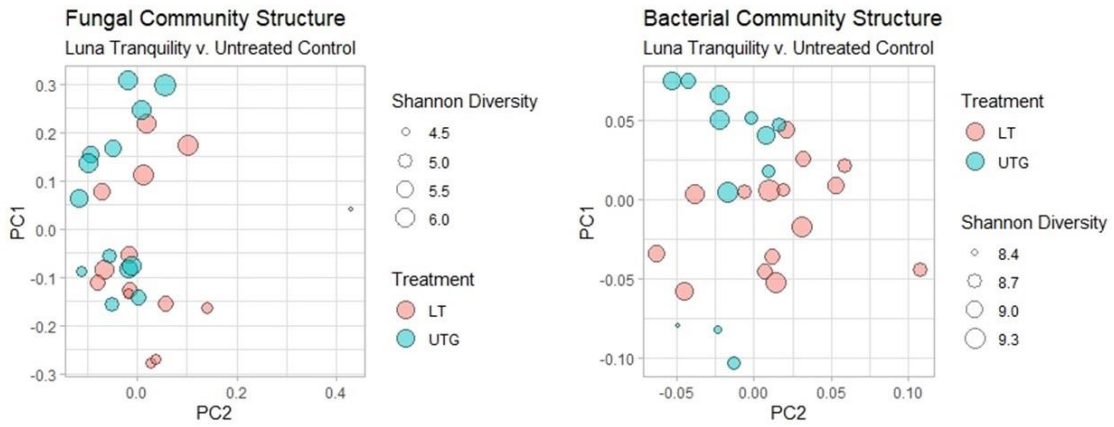

Figure 7: PCoA of community structures, comparing Luna Tranquility (LT) and Untreated Control (UTG) for both the fungal and bacterial populations. In both populations, significant groupings are apparent between Lunar Tranquility and UTG.

4.4.5 Differential taxa relative abundances

4.4.5.1 Bacterial relative abundances

Considering the effect of treatment on the dataset as a whole, a number of bacterial taxa at multiple taxonomic levels were found to differ significantly in relative abundance ($q < 0.1$). At the highest level, three bacterial divisions, Dependentiae, Chlamydiae, and Chloroflexi, varied significantly in relative abundance as a result of treatment groups ($q < 0.1$) (F**igure 9**). Moving down the taxonomic hierarchy, two classes, seven classes, and eleven families demonstrated significant variance in relative abundance as a result of treatment ($q < 0.1$) (F**igure 9**). While the ALDEx2 test found significant variance in relative abundances at the species level, limitations in resolution prevented meaningful relative abundance at this level.

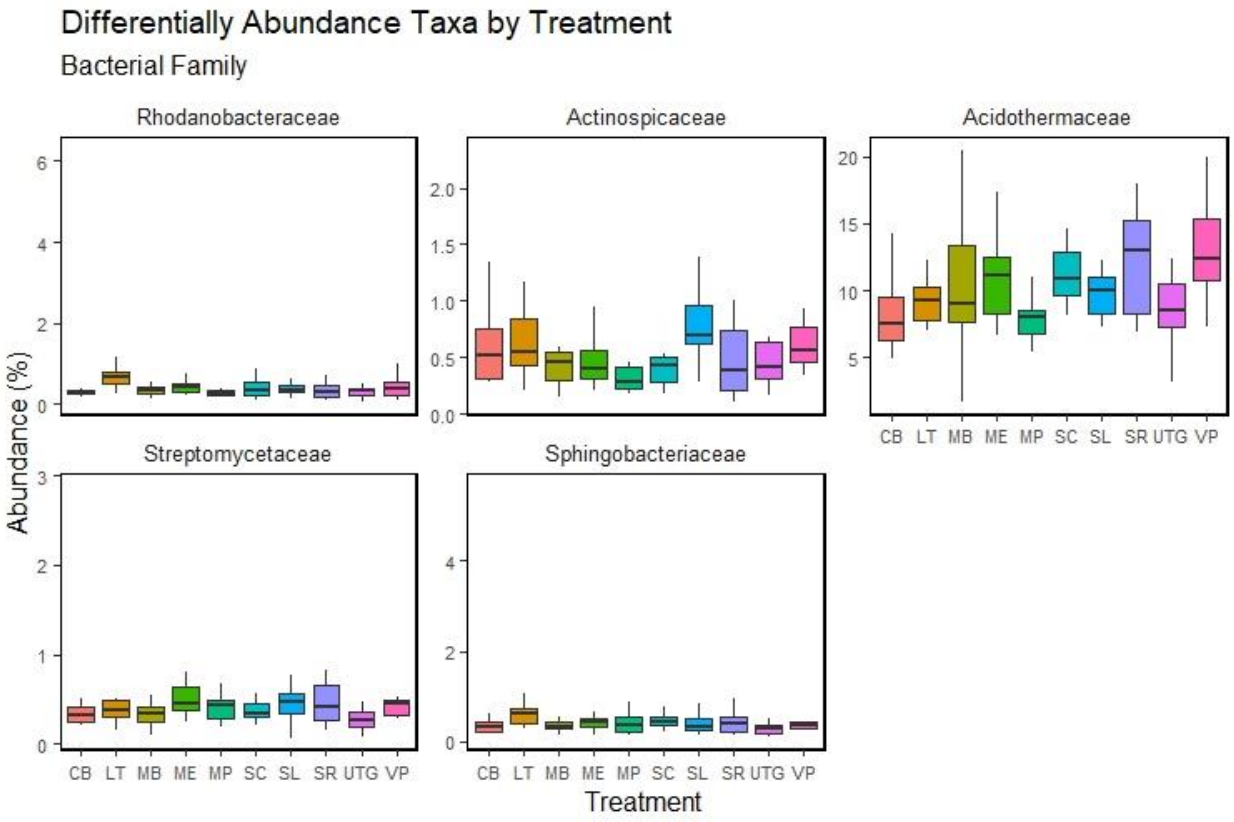

Figure 8: The five most abundant bacterial families whose relative abundances differed significantly as a result of treatment. Abbreviations above: CB – Cabrio; LT – Luna Tranquility; MB – Miravis Bold; ME – Merivon; MP – Miravis Prime; SC – Scala; SL – Scholar; SR – Sercadis; UTG – Untreated Control; VP – Velum Prime.

Comparing Luna Tranquility and Scholar, the pairing with the largest UniFrac variance attributable to treatment, revealed a number of significant differences in relative abundances at all taxonomic levels. One division, Chloroflexi, was found to have significantly reduced relative abundance in Luna Tranquility relative to Scholar ($q < 0.1$). At the order level, which was where the greatest number of significantly differential taxa were found, 13 orders were found to differ between Luna Tranquility and Scholar ($q <$

0.1). Additionally, nine bacterial families, four genera, and two species level taxa were identified as varying significantly between Scholar and Luna Tranquility ($q < 0.1$) (**Figure 8**). Similarly, a large number of taxonomic relative abundances were found to differ significantly between Luna Tranquility and Miravis Prime. In this comparison, 3 divisions, 4 orders, 21 classes, and 18 families differed significantly in relative abundance between Luna Tranquility and Miravis Prime.

Breaking with the trend of Luna Tranquility being the catalyst of significant variance, the pairing of Miravis Prime and Velum Prime generated a great number of significant taxa. Comparing these two groups yielded 22 species, and 25 genera whose relative abundances differed significantly between the two treatment groups ($q < 0.1$). Additionally, a comparison of Miravis Prime and Sercadis yielded some, albeit fewer, significant taxa, with seven families which differed significantly between the two treatments ($q < 0.1$) and significant taxa at all levels except for genus.

4.4.5.2 Fungal relative abundances

Three clades, four orders, and five families differed significantly as a result of treatment ($q < 0.1$) (**Figure 9**). Comparing the treatments in a pairwise manner found numerous taxa which differed significantly in relative abundance. In accordance with the significant variance between UniFrac matrices, these differential relative abundances centered around Luna Tranquility. One family, Clavicipitaceae was found to differ significantly between Luna Tranquility and control (UTG), with a significantly increased relative abundance in Luna Tranquility relative to UTG. The pairing which yielded the

greatest number of differential taxa was the comparison between Luna Tranquility and Scala. At the genus level, five genera differed significantly with two having a decreased relative abundance in Luna Tranquility, with three having an increased relative abundance in Luna Tranquility, relative to Scala ($q < 0.1$). In comparing Miravis Bold and Luna Tranquility, four orders had a significant difference in relative abundances, with two being increased in relative abundance in Luna Tranquility relative to Miravis Bold and two being decreased in relative abundance. Finally, comparing Miravis Prime to Luna Tranquility revealed that one class, Tremellomycetes, had an increased relative abundance in Luna Tranquility relative to Miravis Prime ($q < 0.1$).

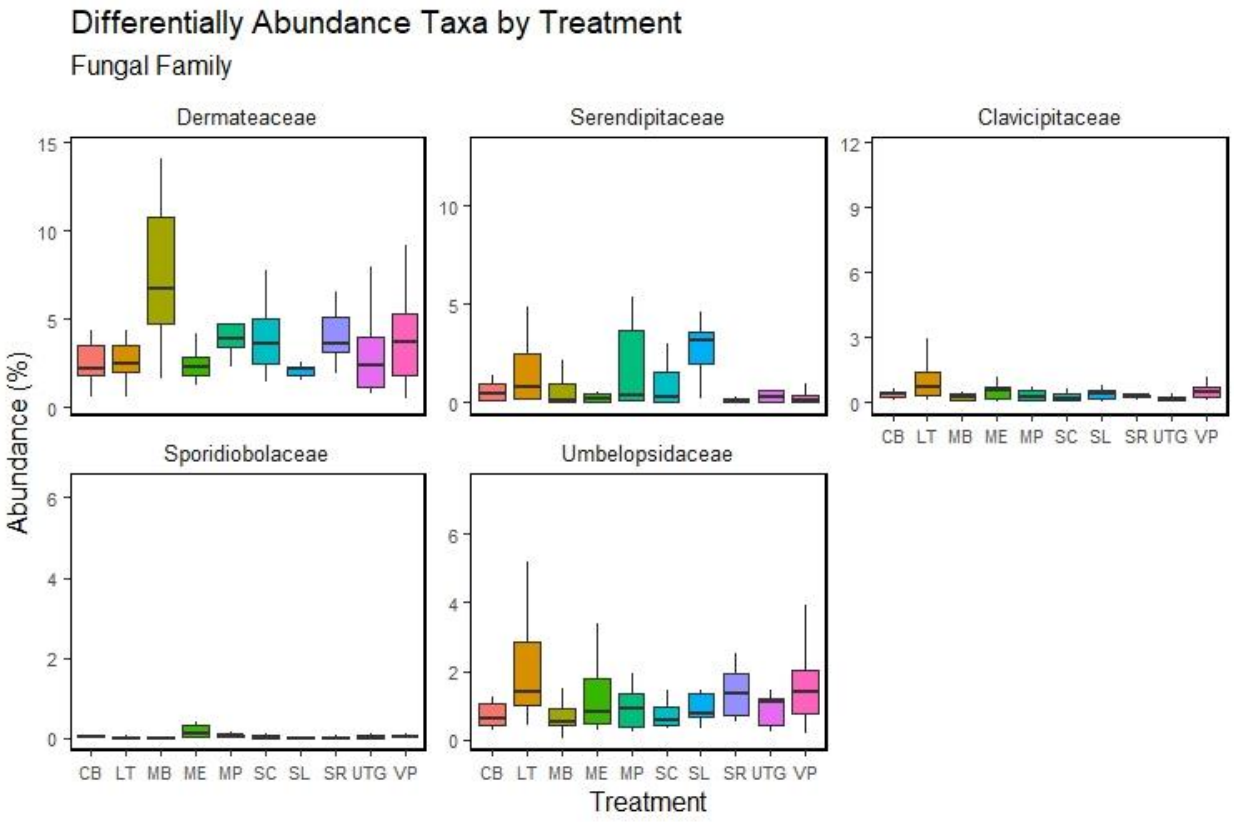

Figure 9: The five most abundant fungal families whose relative abundances differed significantly as a result of treatment. Abbreviations above: CB – Cabrio; LT – Luna Tranquility; MB – Miravis Bold; ME – Merivon; MP – Miravis Prime; SC – Scala; SL – Scholar; SR – Sercadis; UTG – Untreated Control; VP – Velum Prime.

4.4.6 Functional Potential

Fifteen pathways varied significantly as a result of treatment ($q < 0.1$). Many of the significant differences in functional potential centered around Luna Tranquility. Significant differences were found between Luna Tranquility and Sercadis, Scholar, Miravis Prime, Merivon, and UTG ($q < 0.1$). Luna Tranquility differed significantly from UTG with 22 pathways having significantly different relative abundances between the two

groups. The greatest number of differences was between Luna Tranquility and Scholar, with 38 pathways differing significantly in relative abundance between the two treatment groups. Additionally, nine pathways differed significantly between Velum Prime and Miravis Prime ($q < 0.1$).

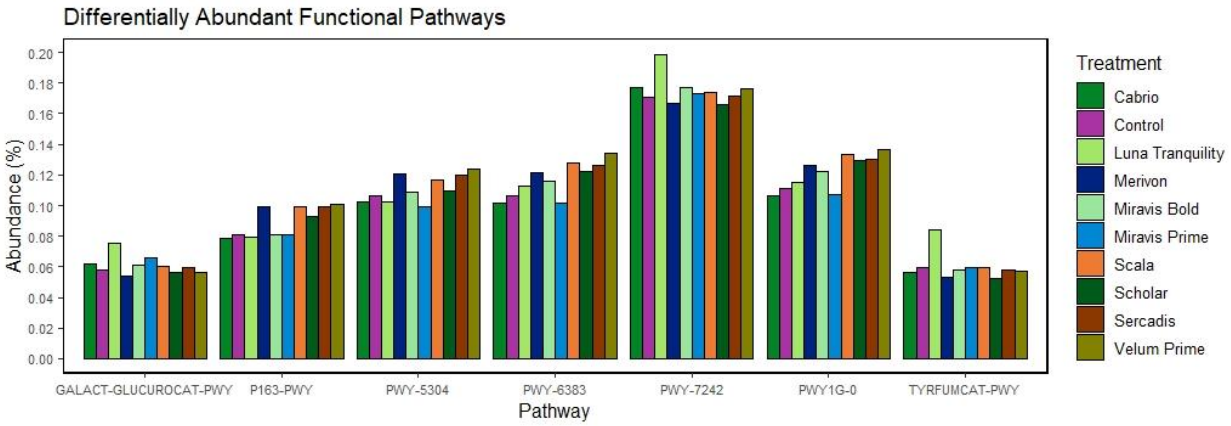

Figure 10: Mean abundances of functional pathways which differed significantly by treatment, the seven most relatively abundant are shown.

4.5 Discussion

4.5.1 Microbial Diversity

The ITS2 data indicated that no significant changes to fungal species richness or evenness occurred as a result of any specific treatment. Additionally, even when comparing CTG and UTG, no differences in either richness or evenness were observed. While no specific treatment led to a change in bacterial alpha diversity, bacterial species richness was found to have significantly increased in CTG relative to UTG, while bacterial evenness was reduced in CTG. These results are not without precedent, pyrimethanil

(Luna Tranquility and Scala) has been shown to lead to a temporary increase in bacterial diversity (Ng et al. 2014).

This set of findings on the subject of species richness and evenness are in contrast to those made in CHAPTER 3 in which it was fungal richness and evenness was significantly altered by the application of fungicides, while the bacterial richness and evenness remained unchanged (Lloyd et al. 2021). These differences may suggest a diversity of responses that the soil microbiome may have to the effects of different fungicides (Lloyd et al. 2021). Given that the trial discussed in CHAPTER 3 pertained to sprout year application, and this study is a crop year application, the difference between the two sets of results on fungal diversity may also indicate either differing effects as a result of differing application practices between the two years, or that the mycobiome may differ between the two halves of the cropping cycle leading to differing sensitivities to fungicides.

4.5.2 Microbial Community Composition

In the fungal ITS2 dataset, it was found that nine treatment combination varied significantly on the community level. Seven of those nine combinations involved Luna Tranquility, which differed significantly from UTG as well as six of the fungicide treatments. These results are paralleled in the 16S data where Luna Tranquility differed significantly from UTG as well as five of the other fungicides. In both cases, Luna Tranquility and UTG showed clear groups in PCoA, and was the only fungicide to do so relative to UTG (**Figure 10**). Luna Tranquility contains two active ingredients, fluopyram

and pyrimethanil. Fluopyram is also the active ingredient in Velum Prime, while pyrimethanil is the active ingredient in Scala.

One study which used carbon source utilization to determine community structure found that fluopyram led to significant changes in the overall community structure (both bacteria and fungi) (Zhang et al. 2014b). However, in the context of the bacterial community in this study, the fact that fluopyram did not lead to changes in community structure is consistent with previous research on the compound which found that fluopyram did not alter bacterial community structures in the rhizosphere (Sun et al. 2020). In contrast, bacterial community-level changes corresponding to pyrimethanil have precedent in previous research on pyrimethanil which found that differences in bacterial community structure correlated to interactions between pyrimethanil dosages and rainfall rates (Ng et al. 2014). It is notable that community structural differences from UTG were only significantly correlated with Luna Tranquility and not Scala, despite both of them containing pyrimethanil. These findings suggest that it is an interactive effect of both fluopyram and pyrimethanil that led to these community-level changes.

4.5.3 Taxonomic Profile

4.5.3.1 Bacterial relative abundances

Ten bacterial families were found to vary significantly across all treatment groups. However, when conducting pairwise comparisons of the treatment groups, no taxa differed significantly between UTG and any of the fungicides, and CTG did not differ from UTG at any taxonomic level. That result is broadly consistent with the findings of CHAPTER

3, in which no bacterial taxa differed significantly between UTG and any other comparison group (Lloyd et al. 2021). Previous literature on some of the trial fungicides may have suggested that taxonomic differences between would have been present between some of the fungicide treatments and UTG. Pyraclostrobin, the sole active ingredient in Cabrio and one of two in Merivon, has been associated with significant changes in bacterial taxa in fluvio-aquic soils (Zhang et al. 2019). Additionally, fluxapyroxad, found in Sercadis as well as Merivon, was noted in a study to reduce the ratio of gram-positive to gram-negative bacteria over time based on phospholipid fatty acid profiles (Wu et al. 2015). While those findings may suggest that some significant shifts in taxonomic relative abundance would be found, none were noted in our analysis with either Sercadis or Merivon. Additionally, fluopyram (found in both Luna Tranquility and Velum Prime) has been shown to increase the relative abundances of phosphorous-solubilizing bacteria in the rhizosphere of pepper plants (Sun et al. 2020). Again, no corresponding increases was found in this study, though this research focused on bulk soil and not the rhizosphere.

Though no fungicide differed significantly from UTG in the 16S dataset, there were some significant differences between the fungicide treatments themselves. In particular, two compounds, Luna Tranquility and Miravis Prime were linked to significant changes in bacterial relative abundances. Comparing Luna Tranquility (fluopyram and pyrimethanil) to both Scholar (fludioxonil) and Miravis Prime (pydiflumetofen + fludioxonil) at the family level, it was found that the taxa which differed significantly in relative abundance had an increased relative abundance in Luna Tranquility relative to the other treatment. For instance, 15 out of the 18 families which were significant in comparing Luna Tranquility

and Miravis Prime showed an increase in relative abundance in Luna Tranquility relative to Miravis Prime. In the same fashion, 6 out of the 9 families which differed between Luna Tranquility and Scholar had a higher relative abundance in Luna Tranquility. Looking at the other two significant treatment pairings, a pattern begins to emerge. Of the 26 bacterial families which differed between Miravis Prime and Velum Prime (fluopyram), 22 had a reduced relative abundance in Miravis Prime. Additionally, 5 out of the 7 families which differed significantly between Miravis Prime and Sercadis (fluxapyroxad) had a reduced relative abundance in Miravis Prime. These results suggest that a subset of bacterial families may be sensitive to fungicides which contain fludioxonil and see reduced relative abundances as a result of treatment by fungicides with this active ingredient. These findings are bolstered by the fact that there was significant overlap in those bacterial families whose relative abundance appeared reduced in fludioxonil-containing fungicides, with 10 families having a reduced relative abundance in two or more of these treatment combinations. The most abundant of these families was Acidothermaceae, which comprised over 8% the total bacterial microbiome of each treatment group. The family was found to be most abundant in Velum Prime, encompassing 12.83% of all bacterial taxa present in that sample group. In contrast, Acidothermaceae represented 8.32% of the samples from Miravis Prime. However, despite this pattern of lowered taxonomic relative abundances centered around fludioxonil, no significant differences occurred between fludioxonil and UTG, suggesting that the effects of these compounds are limited.

4.5.3.2 Fungal relative abundances

Where fungal relative abundances were concerned, significant differences in taxonomic relative abundance appear to center around Luna Tranquility. UTG, Miravis Bold, Scala, and Miravis Prime all contained taxa which differed significantly in relative abundance from Luna Tranquility. The fungal family Clavicipitaceae differed significantly between Luna Tranquility and UTG, exhibiting an increased relative abundance in Luna Tranquility. An unidentified genus of this family was also found to be significantly increased in relative abundance in Luna Tranquility relative to UTG. While the unidentified nature of this genus prevents any concrete conclusions, it is worth consideration that one common genus of this family *Metarhizium*, has been identified as both a root endophyte associated with promoting root growth, and an insect pathogen which has been studied for use as a biopesticide (Hunter et al. 2001; Sasan and Bidochka 2012). This difference in relative abundances parallels a finding in CHAPTER 3 in which another taxa representing potential root symbionts, the fungal family Clavaria, was found to have a greater relative abundance in plots treated with prothioconazole (Lloyd et al. 2021). Both of these changes in relative abundance open the door to the possibility that the fungicide has a side benefit to the crop of increasing root symbiosis. These findings suggested that combination of fluopyram and pyrimethanil as active ingredients might have a side benefit to the crop of increasing root symbiosis. Corroborating the notion that a synergistic effect is the driving factor of these significant changes is the large number of fungal taxa which differed significantly between Scala and Luna Tranquility, both of which share the active ingredient pyrimethanil. Despite this shared compound, the two products presented the greatest

number of significantly different taxonomic relative abundances. These differences strongly suggest that it is the synergistic effect of fluopyram and pyrimethanil which leads to large changes in the fungal microbiome, as opposed to either active ingredient individually.

4.5.4 Functional Potential

Luna Tranquility was the only treatment which induced significant differences in relative abundances of MetaCYC pathways compared to untreated soil. Of the 22 pathways which were found to be differentially represented between the treatments, all but one had a higher relative abundance in Luna Tranquility treated soil relative to UTG. 2-nitrobenzoate degradation I pathway involved in microbial metabolism of nitrobenzoates compounds found in many pesticides (Peres et al. 1999), was upregulated by Luna Tranquility application. Another pathway overrepresented in Luna Tranquility treated soils, PWY-6210, is involved in degradation of 2-aminophenols, common components of pesticides (Bhat and Gogate 2021). The fact that these two pathways governing the degradation of nitrogenous pesticide-associated compounds were increased in relative abundances in soils treated with Luna Tranquility suggests that bacterial bioremediation may be a response to the treatment. In a similar manner, our previous study found that the fungicides applications were associated with an increased relative abundance of enzymes and pathways which degraded halo-organic and xenobiotic compounds (Lloyd et al. 2021). One potential consequence of this bioremediation may be a reduced efficacy of the pesticide. If bacteria are increasing the speed with which the active ingredients are degraded, the span of time during which the plant is protected from pathogens may be reduced (Arbeli and Fuentes

2007). It is thus possible that Luna Tranquility may have a reduced window of protection relative to the other fungicides examined.

Previously it was reported that direct soil application of fluopyram stimulated phosphorous-solubilizing bacteria (Sun et al. 2020). Our study did not find a corresponding change to phosphorous-solubilizing function in the soil after applications of Luna Tranquility, suggesting that other environmental factors, as well as significantly reduced fungicide-soil contact, may drastically alter the effect of fungicide on the soil microbiome and its functions.

4.5.5 Plant Growth and Development

4.5.5.1 Crop Development and Outcomes

The failure of any of the fungicide treatments to distinguish themselves in terms of disease prevention may be attributed to a generally low disease prevalence throughout the dataset on the whole. Only 27 out the 539 stems sampled presented with *Monilinia*, and only one showed signs of *Botrytis*. Results such as these may be the result of a season not conducive to pathogen development. These conditions prevent the drawing of any conclusions on the efficacy of the fungicides tested in combatting disease pressure in this investigation.

In terms of crop outcomes, no significant differences were found in terms of the number of ripe fruits per stem at harvest time. Significant differences in floral development were found at the early July sampling date. Cabrio, Scala, and Miravis Prime were all significantly delayed in floral development, relative to UTG at this sample date,

while no treatment significantly advanced floral development at this stage. However, given that no significant variance was found in either the number of ripe berries or the number of unmarketable berries from one treatment to the next, these significant delays in fruit development relative to untreated plants may not have a deleterious effect on harvest outcomes.

Stem length was also found to vary significantly as a result of treatment. Scala was found to have the shortest stems of any of the treatment groups, significantly shorter than UTG as well as five of the other fungicide treatments. As was the case with floral development timing however, these differences in stem length do not appear to correspond to any effect, positive or negative on the amount of ripe berries at harvest time.

4.5.5.2 Crop outcomes and microbiome effects

The treatment which elicited the greatest number of changes to the soil microbiome relative to both UTG and other treatments, Luna Tranquility, did not have so dramatic of an effect on crop outcomes. No significant changes in floral development timing corresponded to Luna Tranquility. While Luna Tranquility differed significantly in terms of stem length from Scala, it did not differ significantly from UTG or any other treatment group. These findings, combined with observation of no significant difference in ripe berries at harvest time, suggest that the significant microbiome effects apparently caused by Luna Tranquility do not have an immediate effect on crop outcomes.

Furthermore, Scala, the treatment associated with the most significant effects on crop growth and development, did not have a significant effect on 16S or ITS2 community composition. Results such as these suggest that fungicide-associated changes in plant development are not a result of changes to the soil microbiome. However, given that these plant development observations and soil samples were taken from plants the same year as the fungicide application, they do not necessarily indicate that the application of these fungicides will have no effect on crop outcomes or soil health in the long term.

4.6 Conclusion

It is well-established that plants are supported by a wide array of beneficial fungi and bacteria. These organisms should be seen as a critical component of crop and soil health and understanding them and the ways in which they interact with agricultural practices may prove to be a key component of preserving agricultural resources. While the fungicides examined in this trial, taken as a whole, reduced bacterial diversity, one fungicide, Luna Tranquility, appeared to elicit the greatest number of changes to both the bacterial and fungal microbiome. Luna Tranquility led to significant changes in the composition of the bacterial and fungal communities relative to untreated soil, and also led to significant changes in bacterial function. Though these changes are disquieting, Luna Tranquility did not appear to effect in a significant way the crop outcomes analysed in this study, while Scala did, despite not generating significant microbiome effects. The ambiguities presented in these findings suggest a complicated relationship between the microbial ecosystem and the crop that they support. Further research on the interactions between this system and pesticides is needed to attain a more complete idea of how best

to safeguard soil health without sacrificing the agricultural productivity that agrichemicals allow.

CHAPTER 5: FINAL CONCLUSION

The use of microbiome methods to understand soil health is a relatively new front in the struggle for sustainability in agriculture. Advancements in this field have given a great deal of clarity on the myriad ways in which plants and microbes depend upon one another to survive and thrive. The lowbush blueberry, being a native, perennial crop managed from wild populations, has an intimate relationship with its surrounding microbiome and has specialized symbionts which allow it to prosper in acidic, nutrient-poor soil. As a result, the lowbush blueberry holobiont is an important subject of study, with microbiome sequencing being a critical tool in understanding this ecosystem. Previous research has done a lot to uncover the diversity of soil organisms which interact both with each other and the plant, across several distinct ecological niches associated with the plant roots. Additionally, it has been well documented that symbiosis with soil microbes is a critical component of plant health, especially in the case of lowbush blueberries with their specialized ericoid mycorrhizae. However, as has been demonstrated in the studies described above, foliar fungicide application to the crop can have significant effects on the soil microbiome, in terms of diversity, community structure, and species relative abundances for both bacteria and fungi. Furthermore, in both studies changes in bacterial function were found to result from fungicide applications. The effects of these fungicides, however, were not universal. As the second study demonstrated, the majority of the significant microbiome effects recorded relative to untreated soil were attributable to one fungicide. That finding suggests the possibility that, with proper information, farmers may be able to choose fungicides for their crop which pose minimal threat to the health of

their soil. Given the temporal constraints of these studies, it cannot be said what the long-term effect these fungicides might have on the health of the soil. However, the significant changes seen in one season's use suggest that both caution and further research on the subject are necessary.

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