Community Dynamics of the Phyllosphere Microbiome

by

Destiny Brokaw

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
May 7, 2022

Keywords: *Xanthomonas*, microbiome, phyllosphere,
mixed infection, disease severity

Approved by

Neha Potnis, Professor of Plant Pathology
Leonardo De La Fuente, Professor of Plant Pathology
Di Tian, Professor of Crop, Soil, and Environmental Sciences

Abstract

The phyllosphere of a plant creates a complex niche for inhabiting microorganisms in which interactions will occur. The dynamics of the interactions between a foliar plant pathogen, *Xanthomonas perforans*, and the resident microflora are assessed in this study. The community dynamics assessed in this study include dysbiosis, type VI secretion system (T6SS) utilization and its influence on the phyllosphere microbiome, intraspecific diversity, and disease severity. With a combination of culture-dependent and culture-independent approaches, this study evaluated dysbiosis events between phyllosphere residents and *X. perforans*. This study suggests that the presence of T6SS creates a shift in the overall composition of the phyllosphere microbiome in the early colonization of the pathogen. Further evaluation of intraspecific diversity observed at the field-level across Alabama were implemented in greenhouse conditions to determine whether co-infection of multiple genotypes lead to higher disease severity. This study reveals that mixed infections of one or more genotypes of *X. perforans* leads to higher disease severity.

Acknowledgements

I express my gratitude to my major advisor, Dr. Neha Potnis, for her assistance and support as I navigated graduate school amid a pandemic. I also express my appreciation to my committee members Dr. Leonardo De La Fuente and Dr. Di Tian for their inquiries and support of my studies here at Auburn. I express my thanks to all of my lab members, Dr. Prabha Liyanapathirange, Dr. Michelle Pena, Rishi Bhandari, Auston Holland, Shreya Sadhukhan, and Kylie Weiss who have been my guides, support, and friends throughout my course of study.

Finally, I would like to express thanks to my friends and family who have been my support and encouragement. I especially thank my best friend and best boy, Winston Kite, for his companionship and for enduring long nights of writing.

I dedicate this thesis to my aunt, Teena Massey Hartman, who lost her battle with COVID last November.

To God be all the glory.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

BLS	Bacterial leaf spot
T6SS	Type VI Secretion System
DI	Deionized
PCA	Principle component analysis
NMDS	Non-metric multidimensional scaling
OTU	Operational taxonomic unit
WT	Wild-type
SC	Sequence cluster
AUDPC	Area under the disease progress curve
LM	Linear model
LMM	Linear mixed model
TALE	Transcription activation-like effector
CI	Confidence interval

1. CHAPTER ONE

Introduction and Literature Review

Introduction

Disease development in plant has historically been referred to as a three-way relationship between the plant host, the pathogen, and the environment (Rastogi *et al.* 2013). However, this disease triangle is missing the microbiota which is a key component to the establishment of the pathogen (Rastogi *et al.* 2013). While our understanding of microbial dynamics has grown, this knowledge is limited to certain groups of microorganisms, and the dynamics and interactions of major groups of the phyllosphere microbiota are still unknown (Bashir *et al.* 2021).

While the phyllosphere supports a large diversity of microbial organisms, there is little information regarding colonization and survival of plant pathogenic and beneficial bacteria (Saleem *et al.* 2017). Early studies of the phyllosphere microbiome are essentially focused on bacteria and driven by plant pathogen inquiries, however most phyllosphere inhabitants live as commensals to the host plants and are much less understood and studied (Müller and Ruppel 2014; Rastogi *et al.* 2013; Senthilkumar and Krishnamoorthy 2017). These commensals are theorized to play a large role in the cycling of elements, remediating pesticides and hydrocarbon pollutants, or are considerably relevant for plant health as biofertilizers, biopesticides/ biocontrol agents against pathogens, and phytostimulators (Müller and Ruppel 2014). Studies have furthered the notion that commensal microbiota of the leaves can play a role in the exclusion of the pathogen and can overall contribute to plant health and productivity (Rastogi *et al.* 2013).

While environmental microorganisms may not be suitable for nutrient media, Müller and Ruppel suggest that microorganisms coined nonculturable would be better defined as being not

yet cultured (2014). Culture-dependent methods of profiling the phyllosphere tends to miscalculate diversity, however culture-independent approaches like 16S rDNA sequencing of the entire microbial community provides a more complete and complex community structure (Sivakumar *et al.* 2020). These molecular studies suggest Proteobacteria and Firmicutes as the dominant phyllosphere inhabitants, along with frequently occurring Acidobacteria, Actinobacteria, and Cyanobacteria (Sivakumar *et al.* 2020).

Some studies have utilized first-generation molecular techniques; however, these results have low throughput and provide only a superficial view of the microbial communities (Rastogi *et al.* 2013; Sivakumar *et al.* 2020). The introduction of next-generation sequencing has allowed for higher throughput which allows the multiplexing of many hundreds of samples in one sequencing run which has allowed for more omic exploration (Rastogi *et al.* 2013; Sivakumar *et al.* 2020). Using "-omic" techniques, further guidelines and directions have been drawn in which the entire plant-associated microbial community must be regulated and evaluated to produce probiotics for plants (Müller and Ruppel 2014). The application of next-generation technologies in the phyllosphere microbiome are significantly increasing, allowing for more depth and insight into community variation, functions, drivers, and interactions (Rastogi *et al.* 2013). Procedures to analyze the phyllosphere's microbial composition has been limited due to bias of cultivation based on 16S rRNA amplicon sequencing which may be subject to inhibitory compounds, primer mismatches, and the amplification of plant-derived RNA (Müller and Ruppel 2014). Whole metagenome shotgun sequencing allows for high-throughput genome analyses which can provide information on individual genes or organisms (Müller and Ruppel 2014). As new technologies and techniques develop, the species richness of the phyllosphere community has extended considerably to less abundant/occurring taxa (Müller and Ruppel 2014).

Plant beneficial microbes have agricultural importance as they can be utilized in plant growth stimulation, plant nutrient uptake nitrogen fixation, siderophore production, increasing crop yields, contaminant removal, and pathogen inhibition (Sivakumar *et al.* 2020). Many bacterial inhabitants of the phyllosphere are being pursued as sources for biological control agents for biotechnological application (Zhang *et al.* 2010). Studies of phyllosphere epiphytes however are highly biased due to models taking place under non-natural conditions (Senthilkumar and Krishnamoorthy 2017). Therefore, in objective one of this study, our model system was exposed to natural condition to extend our focus beyond the greenhouse. The central focus of this study is further our understanding of the community dynamics on the phyllosphere microbiome. Our model system for this study is tomato and our model organism, *Xanthomonas perforans*, in order to have a comprehensive understanding of the varying interactions occurring on the phyllosphere microbiome.

Xanthomonas perforans and bacterial leaf spot

Tomato, *Solanum lycopersicum*, is a large contributor to the agricultural industry and serves as a great model system in basic and applied research (Dong *et al.* 2019; Enya *et al.* 2007). Tomato produces a saponin compound, a-tomatine which has antimicrobial activity and is a determining factor in phyllosphere colonization and structure (Enya *et al.* 2007). One of the main issues with the tomato industry is the control of diseases, therefore the characterization of the bacterial communities on tomato contributes to potential biocontrol control candidates and application strategies (Dong *et al.* 2019).

Bacterial leaf spot (BLS) of tomato is a disease observed worldwide that results in high yield loss and is often caused by *Xanthomonas perforans*, a yellow- pigmented, rod-shaped,

gram-negative, single-flagellated, aerobic bacteria (Abrahamian *et al.* 2021). BLS has four main causal agents which include: *X. perforans*, *X. euvesicatoria*, *X. gardneri*, and *X. vesicatoria* (Abrahamian *et al.* 2021; Timilsina *et al.* 2016; Potnis *et al.* 2015). Xanthomonads are largely known for their pathogenicity on specific host plants, however, tend to be looked over as commensals on other hosts (Jacques *et al.* 2016). *X. perforans* has been reported worldwide in areas such as Canada, Australia, Ethiopia, Brazil, Italy, Korea, Iran, Nigeria, Mexico, Tanzania, Taiwan, Thailand, and the United States (Alabama, Florida, Georgia, Illinois, Indiana, Louisiana, Mississippi, North and South Carolina, Ohio) (Abrahamian *et al.* 2021). *X. perforans* tends to thrive in high humidity and high temperature conditions and disseminate through rain splash (Abrahamian *et al.* 2021). Classical symptoms of BLS include necrotic lesions with frequent yellow halos surrounding the border edges on the leaves, stems, pedicels, sepals, and fruits along with water-soaked lesion when wet (Abrahamian *et al.* 2021; Potnis *et al.* 2015). *Xanthomonas* colonize the plant surfaces/ phyllosphere before entering the plant tissues/ mesophyll through openings such as stomata, hydathodes, lenticels and wounds (Jacques *et al.* 2016). It has been hypothesized that metabolite production of *Xanthomonas* can modify the composition of the phyllosphere microbiota, but its influence has yet to be determined (Jacques *et al.* 2016).

The phyllosphere microbiome

Plants have a limited area in which they must evolve strategies to withstand due to the inability to move, therefore plants have become more reliant on coevolution processes between microbes and their surrounding environment (Lemanceau *et al.* 2010). Microorganisms surrounding the plant are abundantly promoted through the release of organic compounds from the plant and can thrive in three main regions of the plant known as the spermosphere,

rhizosphere, and phyllosphere (Lemanceau *et al.* 2010). The phyllosphere is the term coined for the complete above ground surfaces of a plant which can serve as a habitat for microorganisms, epiphytes, ranging from bacteria, yeasts, filamentous fungi, archaea, and protists that have adapted to nutrient and water- limiting environments (Zhang *et al.* 2010; Müller and Ruppel 2014). Bacterial inhabitants may include pathogenic or non-pathogenic organisms which may be further involved in influencing the environment, host health, and the carbon and nitrogen cycles (Zhang *et al.* 2010).

Bacterial communities of the phyllosphere microbiome

The phyllosphere microbiome has been largely influenced and observed in recent years due to the development of large-scale sequencing technology and the advancement of bioinformatics, enabling studies of the plant microbiota to fill in previous literature gaps (Gong and Xin 2021). In comparison to rhizosphere microbiota studies, the phyllosphere microbiota has experienced a delay in research until recently (Gong and Xin 2021). Many profiling studies using next-generation sequencing, have cataloged a diverse bacterial community profile for the phyllosphere microbiome including Proteobacteria, Actinobacteria, and Bacteroidetes across varying plant species (Gong and Xin 2021). Sources of the phyllosphere microbiota can range from seeds, soil, and air in which communities can further interact via wind, rain, or insect dispersal, however the relative contribution of each source is unknown (Gong and Xin 2021; Rastogi *et al.* 2013; Bashir *et al.* 2021; Sivakumar *et al.* 2020). Studies have shown that while the leaf and root microbiomes overlap, that a distinctive community structure can be determined overall (Gong and Xin 2021). Studies of the rhizosphere microbiome have determined a sequential assembly and understanding of how the microbial communities are governed,

however, these studies of phyllosphere microbiome assembly are lacking (Gong and Xin 2021). Results suggest that interactions among strains and the arrival of the colonizer, also referred to as priority effects, influence the microbial community structure (Gong and Xin 2021).

Research has indicated that the soil microbial community is a significant source of the leaf microbiota in which studies have observed the transport of bacterial residents from the soil to the leaf via the plants vascular system (Gong and Xin 2021). Dysbiotic plants have been observed to have a lower alpha diversity in the phyllosphere and an overall shift in the community structure as Firmicutes-rich to Proteobacteria-rich communities which has also been linked to gut microbiota in human diseases (Gong and Xin 2021). In an evaluation of vegetable phyllosphere community structure (celery, spinach, rape, cauliflower, and broccoli), sequences were classified into Proteobacteria, Bacteroidetes, Cyanobacteria, and Firmicutes in which most belonged to the Firmicutes and Proteobacteria groups (Zhang *et al.* 2010). Advancement in culture-independent techniques have revealed that the phyllosphere across a diverse selection of agricultural crops (e.g. rice, wheat, spinach, lettuce, and apple) are largely composed of Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Rastogi *et al.* 2013). Further analyses at a genus level suggests that the phyllosphere is consistently composed of *Pseudomonas*, *Sphingomonas*, *Methylobacterium*, *Bacillus*, *Massilia*, *Arthrobacter*, and *Pantoea* across a diverse range of plant species (Rastogi *et al.* 2013; Sivakumar *et al.* 2020).

Factors such as the host genotype and age, soil type, geography, fertilizer, management practices, temperature, seasonality, moisture, carbon dioxide, light, and nutrients have a significant influence on the phyllosphere community (Gong and Xin 2021; Müller and Ruppel 2014; Lemanceau *et al.* 2010; Rastogi *et al.* 2013; Bashir *et al.* 2021; Sivakumar *et al.* 2020; Dong *et al.* 2019). Some of these factors have been stressors that have caused some bacterial

species to develop DNA repair systems or pigment production in response to UV exposure, extracellular polysaccharide production and aggregation from desiccation (Rastogi *et al.* 2013; Bashir *et al.* 2021). Due to a significant correlation between plant species and the composition of the bacterial community, plant genetic factors are strongly implied to have an influence of the microbiota (Rastogi *et al.* 2013). Residents on the phyllosphere microbiota have varying interactions which include competition via direct inhibition, antibiotic production, and indirect inhibition, plant defense initiation, which are generally motivated for nutrition and space (Gong and Xin 2021; Sivakumar *et al.* 2020; Dong *et al.* 2019). Studies have indicated that phyllosphere residents can enhance the plant's resistance to plant pathogens via antimicrobial production, microbial competition, or plant defense activation (Gong and Xin 2021).

Metagenomic and metaproteomic analyses have revealed up to 4308 varying proteins, with 62% of bacterial and archaeal origin, as well as microbial proteins that are specific for phyllosphere adaptation (Müller and Ruppel 2014). The overall leaf structure along with the grooves, hydathodes, stomata, and trichomes has been observed to play a role in differentiating bacterial populations across the phyllosphere (Müller and Ruppel 2014; Bashir *et al.* 2021). It is suggested that both structural and metabolic differences in leaves can cause for selection of microbial growth on the phyllosphere (Müller and Ruppel 2014). Bacterial colonization is unevenly distributed and are most often observed at the bases of trichomes, stomata, cell wall grooves, and along veins due to the partial shielding from environmental stressors (Müller and Ruppel 2014; Bashir *et al.* 2021; Sivakumar *et al.* 2020). Glandular trichomes release leaf exudates which has been associated with plant-microbe interactions (Sivakumar *et al.* 2020). There is a lack of understanding of how the plant host it is controlling the phyllosphere community structures and is a gap in the literature that is needed to fill in order to move forward

with biotechnological use of the phyllosphere community (Müller and Ruppel 2014). Understanding the mechanisms, genes, and compounds involved between the phyllosphere residents is necessary for biological usage for plant protection (Müller and Ruppel 2014). In a study of the bacterial community composition of leaves in a preharvest field of lettuce it was determined that there was a correlation between the abundance of the pathogen, *Xanthomonas campestris pv. vitians*, and the presence/absence of other phyllosphere bacteria (Müller and Ruppel 2014). It has been hypothesized that the genus *Alkanindiges* can help facilitate the pathogen, while *Bacillus*, *Erwinia*, and *Pantoea* may serve as antagonists of the pathogen (Müller and Ruppel 2014).

Plants in turn are also able to modulate the composition of the microbial communities through communication pathways that rely on trophic interactions and molecular signals (Lemanceau *et al.* 2010). Phyllosphere colonization starts at seedling emergence and are typically acquired horizontally from the surrounding environment, while some can be transferred vertically from the maternal plants via seed offspring (Lemanceau *et al.* 2010; Bashir *et al.* 2021). The leaves of the plants select microbial communities of certain microorganisms which can thrive is known as the phyllosphere effect, this further distinguishes the phyllosphere community from airborne communities (Lemanceau *et al.* 2010). Airborne and rhizosphere communities have been shown to heavily influence the phyllosphere microbial community, however the leaf has also shown selection for microbial species in these cases (Lemanceau *et al.* 2010). The structure and composition of the leaf cuticle has significant influence on the phyllosphere microbial communities based on the ability of the microorganisms to attach to the leaf cuticle (Lemanceau *et al.* 2010; Sivakumar *et al.* 2020). Intriguingly, the presence of trichomes does not have influence on the microbial composition and leaf size does not influence

microbial diversity (Lemanceau *et al.* 2010). The presence of certain foliar nutrients is expected to have a major influence on the microbial communities of the phyllosphere, hence studies have shown that leaf nitrogen content is correlated the structure of phyllosphere microbiota across plant species (Lemanceau *et al.* 2010). Plant loci for defense and cell wall integrity have been shown to be drivers for both fungal and bacterial communities of the phyllosphere in *Arabidopsis thaliana* (Lemanceau *et al.* 2010). Microbial adaptation to the phyllosphere include nutrient acquisition, leaf cuticle adhesion, and biotic/ abiotic stress tolerance (Lemanceau *et al.* 2010). Microbial rhodopsins in the phyllophere suggest that some microorganisms have evolved phototrophic ability to overcome nutrient scarcity, while some microorganisms, such as *Pseudomonas syringae* which synthesize phytotoxins to regulate stomatal aperture, have evolved the capability to enter the leaf apoplast for a more nutrient rich environment (Lemanceau *et al.* 2010).

The majority of leaf colonizers are bacteria, in which most epiphytic bacteria are commensal (Rastogi *et al.* 2013). Internal interactions of the microbes, commensal, antagonistic, or mutualistic associations help to shape and determine the overall community structure (Sivakumar *et al.* 2020). Commensal strains have weak interactive partners and are randomly dispersed (Sivakumar *et al.* 2020). Some of the bacterial species may serve as biogeochemical cyclers, phytoremediators of toxic pollutants, while other may contribute to pathogen exclusion or plant probiotics (Rastogi *et al.* 2013). In a study comparing the phyllosphere and rhizosphere microbiota for unique metabolic processes, it was observed that the phyllosphere of rice supported large populations of *Rhizobium*, *Methylobacterium*, and *Microbacterium* along with proteome analyses indicating methylotrophic enzymes (Rastogi *et al.* 2013; Sivakumar *et al.* 2020). Genes typically associated with nitrogen fixation were found in both the phyllosphere and

rhizosphere samples, however proteomic analysis indicates that expression in found only within the rhizosphere (Rastogi *et al.,* 2013; Sivakumar *et al.* 2020). Major phyllosphere inhabitants such as *Bacillus*, *Burkholderia*, *Methylobacterium*, *Pseudomonas*, *Sphingomonas*, and *Xanthomonas* share carbohydrate metabolizing properties of the rhizosphere (Sivakumar *et al.* 2020).

It has been observed that Proteobacteria in the most dominant bacterial phylum of the phyllosphere, and in some cases a large portion of Firmicutes, with less frequent Actinobacteria, Acidobacteria, and Cyanobacteria (Bashir *et al.* 2021). Representative members within Proteobacteria are observed to carry out plant functions such as nitrogen fixation, nitrification, and methylotrophy/ anoxygenic photosynthesis (Bashir *et al.* 2021). Reports have been made of nitrogen-fixing microbes in the phyllosphere have been documented in crops such as wheat, rice, mustard, potato, sugarcane, jute, banana, and tomato (Bashir *et al.* 2021). Bacterial residents of the phyllosphere have also been shown to influence the availability of nutrients for the host plant such as the solubization of inorganic, insoluble phosphorous (Bashir *et al.* 2021). Bacterial genera such as *Bacillus*, *Pseudomonas*, *Methylobacterium*, *Acetobacter*, *Enterobacter*, *Arthrobacter*, *Chryseobacterium*, *Delftia*, *Gordonia*, *Klebsiella*, *Rhodococcus*, *Serratia*, etc., have been documented as phosphate solubilizers (Bashir *et al.* 2021). Representative members of Actinobacteria and Firmicutes are typically found in arid conditions and are observed to attributes such as spore production, pigment production, and UV damage repair (Bashir *et al.* 2021; Sivakumar *et al.* 2020). Influence of plant growth by the phyllosphere microbiota has been attributed to many phyllosphere microbes in which can synthesize indole-3-acetic acid, a precursor of the plant hormone, auxin, as well as cytokines, and abscisic acids (Bashir *et al.* 2021; Sivakumar *et al.* 2020). Cytokinins of the host plants trigger the release of methanol and is

thought to be a reason for the abundance of the methanol consuming bacteria, *Methylobacterium*, on the phyllosphere (Bashir *et al.* 2021).

Xanthomonas in the phyllosphere microbiome

The presence of leaf-associated pathogenic xanthomonads have been reported on both hosts and nonhosts (Stromberg *et al.* 2004). Epiphytic population of *Xanthomonas campestris p.v. vesicatoria* has been observed be greater on susceptible cultivars compared to resistant cultivars (Sundin 2006). *Xanthomonas euvesicatoria* has been observed to invade the host plant through stomata, hydathodes, and wounds (Zhang *et al.* 2009). *Xanthomonas euvesicatoria* has been reported to have an epiphytic stage where abundant growth occurs on the leaf surface/ phyllosphere (Zhang *et al.* 2009). Using fluorescent tagging, *Xanthomonas euvesicatoria* was observed to occupy depressions of epidermal cells, guard cells, and in the stomatal opening, however after 72 hours post inoculation, the mesophyll tissue became heavily colonized in contrast to the leaf surface which was not as abundant (Zhang *et al.* 2009). It is suggested that some genes of the HrpG regulon of the type III secretion system may be necessary for bacterial growth on the leaf surface and invasion (Zhang *et al.* 2009). Experiments observing the survival *of Xanthomonas campestris pv. campestris* determined that climatic variables such as rainfall, temperature, and relative humidity played an influence on survival rates on the phyllosphere, however intense and frequent rainfall may reduce bacterial cells from the phyllosphere (Stromberg *et al.* 2004). The adaptability of *Xanthomonas campestris pv. campestris* has been contributed to factors such as xanthomonadine pigment from photooxidative stress, extracellular polysaccharides, and aggregate formation (Stromberg *et al.* 2004). Negative interactions between

phyllosphere bacteria and *Xanthomonas axonopodis pv. dieffenbachiae* have been observed and has shown that several indigenous strains can inhibit the pathogen (Enya *et al.* 2007).

Bacterial phyllosphere residents of tomato

Understanding of the tomato phyllosphere microbiome has significant public health implications, especially regarding *Salmonella* associated outbreaks, therefore the tomato microbiome has become an important system for research (Ottesen *et al.* 2016). Biogeographical and environmental factors have shown to have more significant influence on epiphytic microbes than water sources, pesticide application, and other management practices (Ottesen *et al.* 2016). The influence of seasonality on the phyllosphere microbiome has been demonstrated in both culture-dependent and culture-independent studies (Ottesen *et al.* 2016). Correlations unique to the phyllosphere of tomato was shown among Bacilli and Betaproteobacteria with genera such as *Ralstonia*, *Staphylococcus*, and *Arthrobacter* (Ottesen *et al.* 2016). The overall richness and diversity are found to be significantly lower in the phyllosphere of tomato in comparison to the rhizosphere (Dong *et al.* 2019). Population sizes of field-grown tomatoes tend to be larger in comparison to greenhouse-grown tomatoes, however richness is not significantly different (Enya *et al.* 2007). The richness of the phyllosphere however is observed to be higher than the endosphere and is contributed to the sources of inoculum (Dong *et al.* 2019). A study of both greenhouse- and field- grown tomatoes have revealed a dominance of *Bacillus* and *Pseudomonas* (Enya *et al.* 2007). In one study by Dong *et al.,* only *Acinetobacter* was abundant in their tomato samples, however it has been reported that epiphytes of tomato are largely composed of *Xanthomonas*, *Rhizobium*, *Methylobacterium*, *Sphingomonas*, and *Pseudomonas*. *Methylobacterium spp*. has been observed to colonize both the endosphere and phyllosphere and

is capable of using plant-derived methanol as a carbon source (Senthilkumar and Krishnamoorthy, 2017). *Methylobacterium* has been shown to have a close association with plants in which they can promote plant growth a development through the production of plant hormones such as auxin and cytokinins (Senthilkumar and Krishnamoorthy, 2017). Application of *Methylobacterium* to tomato displayed improvement in plant growth and yield (Senthilkumar and Krishnamoorthy, 2017). A study incorporating samples of tomato and pepper fields in Alabama, revealed correlations of *Xanthomonas* spp. with several opportunistic human pathogens, *Klebsiella* spp., *Enterobacter* spp., and *Citrobacter amalonaticus*, along with plant associated epiphytes, *P. syringae*, *P. oryzihabitans*, *Aureimonas ureilytica*, *Sphingomonas* and *Methylbacterium* (Newberry *et al.* 2020). A high abundance of mOTUs for *Stenotrophomonas maltophilia*, *P. fulva*, *Defltia acidovorans*, and *P. fluorescens* were also identified in these fields (Newberry *et al.* 2020).

Pathogen intraspecific diversity, population, and disease severity

Leaf-associated plant pathogenic bacterial populations can be utilized as a direct source of inoculum under favorable conditions (Stromberg *et al.* 1999). The role of population's influence on disease symptoms are largely unknown for xanthomonads, however is theorized to be positively correlated (Stromberg *et al.* 1999). In one study to observe this correlation, pathogen inoculum concentration determined the amount of time needed for BLS symptoms to initiate; Increasing the inoculum, decreased the time (Stromberg *et al.* 1999). High inoculum of 10^7 and 10^8 resulted in symptom initiating four days post inoculation (Stromberg *et al.* 2004). It was observed that as inoculum concentration increased, the population sizes and disease severity also increased (Stromberg *et al.* 1999). With recent developments in pathogenomics, it is

important to incorporate and consider the extent of intraspecific diversity occurring within the pathogen populations in the development of management strategies (Newberry *et al.* 2020). Disease outbreaks may be incited by varying mixtures of strains throughout agricultural fields (Newberry *et al.* 2020). Profiling strains of single nucleotide patterns can allow for intraspecific diversity within a population to be detected (Newberry *et al.* 2020). A maximum-likelihood phylogeny of *X. perforans* strains isolated from pepper and tomato plants from Alabama and Florida, revealed six distinct sequence clusters (Newberry *et al.* 2019). Using single nucleotide variant profiling, co-occurrence of *X. perforans* genotypes were assessed for individual fields in Alabama and varying combinations of sequence clusters were observed (Newberry *et al.* 2020). Four tomato fields in Macon, Lee, Tuscaloosa, and Dekalb Counties were observed to have a single infection of a *X. perforans* genotype (SC3 or SC5), one Tuscaloosa County field observed co-occurrence of two genotypes (SC3 and SC5), and an observation of three genotypes in fields of Baldwin and Etowah Counties (SC2, SC4, and SC6) (Newberry *et al.,* 2020).

Xanthomonas effectors involved in disease severity

Evaluation of virulence factors within a pathogen is important to assess in how they may contribute to fitness (Newberry *et al.* 2020). Two effectors, XopJ2 and members of the AvrBs3 family, transcription activation-like effectors, are important virulence factors of Xanthomonads (Newberry *et al.* 2020). XopJ2 is shown to be strongly associated with SC3 and AvrBs3 with SC5 (Newberry *et al.* 2020). Sequence clusters SC4 and SC5 are shown to typically carry PthXp1, associated with enhanced symptom development, and SC6 carries AvrHah1, an emergent lineage found to be pathogenic to both pepper and tomato (Newberry *et al.* 2020; Newberry *et al.* 2019). It is hypothesized that a low presence of the AvrBs3 family and XopJ6 is

due to the presence of SC4 (Newberry *et al.* 2020). A recent study comparing a wild-type *Xanthomonas perforans* strain with a mutant on XopJ2, observed that the wild-type bacteria dispersed at a rate three times faster than the XopJ2 mutants (Sharma *et al.* 2021). The fitness established by XopJ2 has been conferred by its faster dispersal velocity of the pathogen, increased field coverage, and increased frequency over generations (Sharma *et al.* 2021). A previous study had also determined that distance was longer from the initial point of inoculation in the wild-type strains compared to the XopJ2 mutant (Sharma *et al.,* 2021). Another important type of effector are the type-III secreted effectors which alter transcription, hijack cell signaling, and suppress host defenses which are transferred into their targeted host (Sharma *et al.* 2021; Timilsina *et al.* 2016). Signaling cascades in xanthomonads are regulated by RpfC/RpfG in quorum signaling (Potnis *et al.* 2015). The HrpG/HrpX system controls the activation of the type-II secretion system and type-III secretion system (Potnis *et al.* 2015). Xanthomonads have been evaluated to have at least 45 effectors, a few core effectors include: AvrBs2, XopD, XopF1, XopK, XopL, XopN, XopQ, XopR, XopX, XopZ1 and XopAD (Potnis *et al.* 2015). XopD, XopL, and XopN are virulence factors known to interfere with pathogen-associated molecular pattern-triggered immunity (Potnis *et al.* 2015).

In this thesis, we planned to address current gaps of the cited literature to study the interactions occurring between *Xanthomonas perforans* and phyllosphere residents of tomato and the effects of intraspecific diversity of *Xanthomonas perforans* when occupying the phyllophere niche. The objectives of this study aim to address dysbiosis of phyllosphere residents from *Xanthomonas perforans*, the effect of *tssM*, a core component of the type VI secretion system, on the phyllosphere microbiome, and the effect of intraspecific diversity on disease severity. The construction of a phyllosphere library and the development of competition assays allowed us to

assess these interactions in a culture-dependent manner, while amplicon sequencing of the phyllosphere microbiome in the presence of a wild-type and *tssM* mutant strain of *Xanthomonas perforans* addressed our questions in a culture-independent manner. Co-infection of tomato with observed genotypes of *Xanthomonas perforans* on a filed level allowed us to distinguish a trend between genotype co-infection and disease severity. This study provides further investigation of the phyllosphere microbiome and the community dynamics occurring in the presence of *Xanthomonas perforans*.

References

Abrahamian, P., Klein-Gordon, J. M., Jones, J. B., & Vallad, G. E. (2021). Epidemiology, diversity, and management of bacterial spot of tomato caused by Xanthomonas perforans. Applied Microbiology and Biotechnology, 105(16), 6143-6158.

Bashir, I., War, A. F., Rafiq, I., Reshi, Z. A., Rashid, I., & Shouche, Y. S. (2021). Phyllosphere microbiome: Diversity and functions. Microbiological Research, 126888.

Dong, C. J., Wang, L. L., Li, Q., & Shang, Q. M. (2019). Bacterial communities in the rhizosphere, phyllosphere and endosphere of tomato plants. PLoS One, 14(11), e0223847.

Dong, C., Wang, L., Li, Q., & Shang, Q. (2021). Epiphytic and endophytic fungal communities of tomato plants. Horticultural Plant Journal, 7(1), 38-48.

Enya, J., Shinohara, H., Yoshida, S., Tsukiboshi, T., Negishi, H., Suyama, K., & Tsushima, S. (2007). Culturable leaf-associated bacteria on tomato plants and their potential as biological control agents. Microbial ecology, 53(4), 524-536.

Gong, T., & Xin, X. F. (2021). Phyllosphere microbiota: Community dynamics and its interaction with plant hosts. Journal of Integrative Plant Biology, 63(2), 297-304.

Jacques, M. A., Arlat, M., Boulanger, A., Boureau, T., Carrère, S., Cesbron, S., ... & Vernière, C. (2016). Using ecology, physiology, and genomics to understand host specificity in Xanthomonas. Annual review of phytopathology, 54, 163-187.

Lemanceau, P., Barret, M., Mazurier, S., Mondy, S., Pivato, B., Fort, T., & Vacher, C. (2017). Plant communication with associated microbiota in the spermosphere, rhizosphere and phyllosphere. Advances in botanical research, 82, 101-133.

Müller, T., & Ruppel, S. (2014). Progress in cultivation-independent phyllosphere microbiology. FEMS microbiology ecology, 87(1), 2-17.

Newberry, E. A., Bhandari, R., Minsavage, G. V., Timilsina, S., Jibrin, M. O., Kemble, J., ... & Potnis, N. (2019). Independent evolution with the gene flux originating from multiple Xanthomonas species explains genomic heterogeneity in Xanthomonas perforans. Applied and environmental microbiology, 85(20), e00885-19.

Newberry, E., Bhandari, R., Kemble, J., Sikora, E., & Potnis, N. (2020). Genome-resolved metagenomics to study co-occurrence patterns and intraspecific heterogeneity among plant pathogen metapopulations. *Environmental microbiology*, *22*(7), 2693-2708.

Ottesen, A. R., Gorham, S., Reed, E., Newell, M. J., Ramachandran, P., Canida, T., ... & White, J. R. (2016). Using a control to better understand phyllosphere microbiota. PLoS one,11(9), e0163482.

Potnis, N., Timilsina, S., Strayer, A., Shantharaj, D., Barak, J. D., Paret, M. L., ... & Jones, J. B. (2015). Bacterial spot of tomato and pepper: diverse Xanthomonas species with a wide variety of virulence factors posing a worldwide challenge. Molecular plant pathology, 16(9), 907-920.

Rastogi, G., Coaker, G. L., & Leveau, J. H. (2013). New insights into the structure and function of phyllosphere microbiota through high-throughput molecular approaches. FEMS microbiology letters, 348(1), 1-10.

Senthilkumar, M., & Krishnamoorthy, R. (2017). Isolation and characterization of tomato leaf phyllosphere Methylobacterium and their effect on plant growth. Int J Curr Microbiol App Sci, 6(11), 2121-2136

Sharma, A., Timilsina, S., Abrahamian, P., Minsavage, G. V., Colee, J., Ojiambo, P. S., ... & Jones, J. B. (2021). Need for speed: bacterial effector XopJ2 is associated with increased dispersal velocity of Xanthomonas perforans. Environmental Microbiology.

Sivakumar, N., Sathishkumar, R., Selvakumar, G., Shyamkumar, R., & Arjunekumar, K. (2020). Phyllospheric microbiomes: diversity, ecological significance, and biotechnological applications. Plant Microbiomes for Sustainable Agriculture, 25, 113.

Stromberg, K. D., Kinkel, L. L., & Leonard, K. J. (2004). Quantifying the effect of bacterial antagonists on the relationship between phyllosphere population sizes of Xanthomonas translucens pv. translucens and subsequent bacterial leaf streak severity on wheat seedlings. Biological Control, 29(1), 58-65.

Sundin, G. W. (2006). Host-pathogen interactions of relevance to the phyllosphere. Microbial ecology of aerial plant surfaces, 201.

Timilsina, S., Abrahamian, P., Potnis, N., Minsavage, G. V., White, F. F., Staskawicz, B. J., ... & Goss, E. M. (2016). Analysis of sequenced genomes of Xanthomonas perforans identifies candidate targets for resistance breeding in tomato. Phytopathology, 106(10), 1097-1104.

Zhang, B., Bai, Z., Hoefel, D., Wang, X., Zhang, L., & Li, Z. (2010). Microbial diversity within the phyllosphere of different vegetable species. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, 2, 1067-1077.

Zhang, Y., Callaway, E. M., Jones, J. B., & Wilson, M. (2009). Visualisation of hrp gene expression in Xanthomonas euvesicatoria in the tomato phyllosphere. European journal of plant pathology, 124(3), 379-390.

2. CHAPTER TWO

Exploration of the Interactions Between *Xanthomonas* spp. and Phyllosphere Residents

Abstract

Plant-pathogen interactions are influenced by several biotic and abiotic factors, among which microbiome component has gained a particular attention in the recent years due to advances in sequencing technologies. The goal of this study is to dissect interactions of the pathogen with the host microbiome to assess their influence on overall host susceptibility. The model pathosystem utilized is a foliar pathogen, *Xanthomonas*, of the host tomato. In this study, the phyllosphere of tomato plants were surveyed through culture-dependent competition assays of isolated bacterial residents from the phyllosphere against *Xanthomonas perforans*. A library of 128 phyllosphere residents, composed of approximately 21 genera, was constructed from tomato fields distributed throughout the Southeastern U.S. and identified through 16S sequencing. A total of 17 phyllosphere isolates inhibited the foliar pathogen, *X. perforans* and contrarily 3 phyllosphere isolates were shown to be inhibited by *X. perforans*. We further dissected these positive and negative interactions to understand the mechanistic basis. Inhibiting and inhibited isolates were evaluated for the involvement of bacteriocins in inhibition of *Xanthomonas*. Prior research from our lab indicates contribution of *tssM*, a core component of Type VI Secretion System (T6SS), towards initial asymptomatic colonization of the pathogen in the tomato phyllosphere. This observation and the well-established research suggesting involvement of T6SS in mediating interbacterial competition in other pathosystems led us to hypothesize that the functional *tssM* of the pathogen is important for creating its niche in the phyllosphere and its ability to overcome competition with resident microflora. In this study, our amplicon data reveals

that the T6SS of *X. perforans* causes a shift in the early colonization of the phyllosphere microbiome. These findings help further our knowledge on how pathogens adapt to/modify the phyllosphere microbiome during host colonization.

Introduction

The plant pathogen, *Xanthomonas perforans*, is a gram-negative, rod-shaped, yellow-pigmented bacteria that is a causal agent of bacterial leaf spot of tomato (Abrahamian *et al.* 2021). *Xanthomonas* is known as a foliar pathogen in which the bacterial cells will colonize of plant surfaces and infiltrate the apoplast via stomata, hydathodes, lenticels, and wounds for infection (Boch and Bonas 2010; Jacques *et al.* 2016; Zhang *et al.* 2009). The phyllosphere is defined as the collective aerial portions of a plant, including stems, leaves, fruits, and flowers. The phyllosphere microbiome refers to a collective community of microorganisms that spatially inhabit the phyllosphere of the plant. Microbial colonizers include epiphytes and endophytes of the plant tissue. Colonizers are typically observed on structures such as stomata, hydathodes, and lenticels where some epiphytic colonizers are able to transition into endophytic colonizers. The phyllosphere microbiome is noted to play a significant contribution to a plant's overall health through the acquisition of its community structure in nitrogen and carbon cycles, defense against foliar pathogens, productivity, and diversification. (Liu *et al.* 2020; Thapa and Prasanna 2018). Consistently observed phyla of the phyllosphere across plant species have included Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes (Liu *et al.* 2020).

Dysbiosis is a term in which a microbial imbalance or loss of microbial diversity is evident due to the abundant presence of a pathogen linking health disparities to the organism. The human gastrointestinal microbiome is analogous to the plant phyllosphere microbiome in

this regard. Plant immune response is dependent on a diversity within the bacterial community composition. This diverse community provides mutualistic interactions and the prevention of pathogen intervention (Liu *et al.* 2020). The most abundant microbial taxonomic groups are typically observed belonging to bacteria and fungi. A lower abundance of organisms such as archaea, protists, and nematodes are also known to reside on the phyllosphere. There is an observed consistency of dominating microbial phylotypes by Bacteroidetes, Actinobacteria, Proteobacteria, and Firmicutes across varying plant species and biogeographical locations. Bacteria are observed to be the most abundant colonizers of the phyllosphere with dominating phyla, Proteobacteria and Firmicutes, and dominating genera, *Bacillus* and *Pseudomonas* (Thapa and Prasanna 2018). Common species cited include *Bacillus*, *Pseudomonas*, *Xanthomonas*, *Sphingomonas*, *Erwinia*, *Acinetobacter*, and *Gluconobacter*. Fungi and yeast are also significant colonizers of the phyllosphere, in which fungi can either inhabit as a saprophyte or phytopathogen. Common genera of foliar fungi include *Penicillium*, *Aspergillus*, *Cladosporium*, *Alternaria*, *Acremonium*, and *Mucor*. Common genera of yeast include *Cryptococcus*, *Sporobolomyces*, and *Rhodotorula* (Thapa and Prasanna 2018). Currently, the role of the foliar pathogen, *Xanthomonas* in dysbiosis of the tomato phyllosphere is not well known. Thus, the construction of a phyllosphere library in this study assesses bacterial diversity on a culture-dependent scale, while amplicon analyses reveal diversity observed in a culture-independent manner.

While there are many missing pieces in the underlying mechanisms utilized by microbial phyllosphere colonizers, there are significant microbe-microbe interactions that are known to shape the microbial composition of the phyllosphere. A key strategy utilized during colonization of the plant's surface is through density-dependent quorum sensing. Molecules released in

quorum sensing ignites a chain reaction of communication with surrounding bacterial cells leading to gene expression of factors ranging from biofilm formation, motility, and virulence. Plant pathogens are typically inhibited through hyperparasitism or competition (Chaudhry *et al.* 2021). Bacterial competition typically utilizes contact-dependent mechanisms including the type VI secretion system (T6SS), antibiotics, and bacteriocins. Bacteriocins are bactericidal effectors in which inter- and intraspecific competition is impacted. Some bacterial species produce antibiotics which are molecules that target different bacterial species or closely related strains. The T6SS is a specialized machinery system regulated by quorum sensing. This system comprises of a bacteriophage-like puncturing device to deliver toxins to the target cell (Shyntum et al., 2019).

The Type VI Secretion system (T6SS) is a specialized machinery system that serves in cell-to-cell contact toxicity (Choi *et al.* 2020; Alvarez-Martinez et al. 2021). The T6SS is a molecular defense mechanism that has been observed in certain bacteria to inject toxins into eukaryotic or prokaryotic neighbors which inhabit the same niche. However, it has not been observed to inject directly into host plant cells. The T6SS complex consists of a compilation of thirteen core components that are typically encoded by a single gene cluster. The T6SS complex has been observed to play an integral role in interbacterial competition between commensal and plant pathogenic phytobacterial strains. Phytopathogens expressing T6SS, are observed to inject toxins in competing bacterial strains. The Type VI Secretion System is often utilized by a variety of bacterial pathogens in competitive niches. Visual assays have been developed in order to determine whether toxins from the T6SS are being secreted into neighboring species. It has been observed that mixed liquid cultures or dropshot methods on Nutrient Agar are inefficient to visually assess this competition. This is likely attributed to the nature of contact-dependency of

the T6SS. Gram-negative bacteria have shown a stronger sensitivity to T6SS activity, however, has not yet been observed in gram-positive bacterial strains (Hachani *et al.* 2013). T6SS has been identified as a key player in inter-bacterial interactions in which the system can be used to deliver proteins directly to bacteria, eukaryotic-host cells, or into its environment (Gallegos-Monterrosa and Coulthurst 2021; Silverman *et al.* 2012). It has been hypothesized that *Xanthomonas* can modify the overall composition of the phyllosphere microbiome, but has yet to be determined (Jacques *et al.* 2016). The role of the T6SS in mediating interactions among phyllosphere residents is not well known in case of *Xanthomonas* and is further addressed in the study through a survey of competition assays between *X. perforans* and phyllosphere residents as well as amplicon sequencing of phyllosphere samples post-inoculation with *X. perforans*.

In *X. perforans*, there are two gene clusters, i3* and i3***, that encode for the functionality of T6SS system (Liyanapathiranage *et al.* 2021). Previous experiments have indicated the role of cluster i3* of *X. perforans* in delaying symptom development on tomato and in asymptomatic epiphytic colonization of the tomato leaf surface (Liyanapathiranage *et al.* 2021). Within cluster i3*, the core gene named *tssM* which encodes for the inner membrane protein of T6SS. A mutant of the *tssM* core gene within *X. perforans* halts the secretion of effector proteins. This mutant will be utilized in this study to assess influence of T6SS. Phyllosphere contact-dependent competition assays were conducted to observe the interaction between phyllosphere isolates and *X. perforans* wild-type and mutant strains.

During epiphytic phase of pathogenesis of *X. perforans*, among several stresses that pathogen has to overcome, competition/ interactions with resident microflora play an important role. Our prior observation of higher epiphytic fitness of cluster i3* carrying *X. perforans* led us to hypothesize that cluster i3* allows pathogen to overcome competition from resident

microflora and thus, alter the phyllosphere microbiome. To test this hypothesis, we have used culture-dependent and culture-independent approaches to identify interactions of *X. perforans* with phyllosphere residents mediated via cluster i3*. The objective of this study is to assess interactions between *X. perforans* and residential microflora of the phyllosphere microbiome and to assess whether inactivation of cluster i3* in the genome of *X. perforans* alters the phyllosphere microbiome composition, which would indicate the influence of T6SS in mediating interactions with the phyllosphere microflora during its epiphytic colonization.

Materials and Methods

Phyllosphere Library Construction

Bacterial isolates were obtained through a culture dependent approach from tomato leaves of fields throughout southeastern United States and the Auburn greenhouse. Samples were placed in a Ziplock bag with 50 mL of wash buffer and sonicated for 25 minutes proceeded by shaking for 25 minutes. The leaf washings were transferred to 50 mL flacon tubes and underwent centrifugation for 25 minutes at 1900 rpm at 4°C. The supernatant was discarded, and the remaining pellet was resuspended in deionized (DI) water and transferred to a 1.5 mL centrifuge tube. The mixed solution was plated on Nutrient Agar with Kanamycin and incubated for 2-3 days. Varying bacterial colonies were transferred to new Nutrient Agar plates. Bacterial pure cultures were stored in 30% glycerol stocks at -80°C.

Contact-Dependent Competition Assays

Bacterial phyllosphere isolates were streaked from frozen culture onto nutrient agar and incubated at 28°C for 1-2 days. *Xanthomonas perforans* Wild-type and Mutant strains were

streaked onto Nutrient Agar with Streptomycin (Wild-type) and Nalidixic Acid (Mutant). Nutrient Agar plates were prepared and outlined with two adjacent circles 2 cm in diameter in sharpie on the plastic of the petri dish. One circle was inoculated with *X. perforans* and the second adjacent circle was inoculated with a phyllosphere isolate. The plates were incubated for five days and observed for neutral, inhibiting, or dominating interactions between the two bacterial strains.

Greenhouse trials to observe contribution of *Xanthomonas perforans* and *tssM*, a core gene of the T6SS cluster i3*, to alteration/manipulation of the plant's phyllosphere microbiome.

Tomato plants were grown in greenhouse conditions and supplied daily with water. Genomic DNA extractions were conducted at two and four weeks of the tomato plant's seedling stage and before inoculation. The four-week-old tomato plants were dip inoculated in .01M $MgSO_4$ buffer with .3 OD of AL65. The variables included the wildtype and cluster i3*strains of *Xanthomonas perforans*. Genomic DNA extractions were conducted after inoculation at one and two weeks and plated for single colony growth. To extract the microbial community from the phyllosphere, 10 grams of leaf tissue or the entire aerial portions of the plant were placed in a Ziplock bag with 50 mL of a phosphate-buffered saline solution amended with .02% tween 20. The bag was sonicated for 25 minutes proceeding shaking for 25 minutes. The washings were then centrifuged at 4°C at 3900 rpm for 25 minutes and the supernatant was discarded. 1.5 mL of DI water was added to the resulting pellet and vortexed. The resulting solution was then transferred to centrifuge tubes where 100 µl of was spread onto Nutrient Agar plates and incubated at 28°C for four days. This culture-dependent approach was used to obtain bacterial

isolates by plating dilutions from the pellet obtained from the leaf washings and identifying colonies showing unique shape, size, and color. The remaining solution underwent centrifugation for three minutes at 19000 rpm at room temperature to continue the genomic DNA extraction. The supernatant was discarded, and 1 mL of DI water was re-added with repeated centrifugation for 3 minutes at 19000 rpm. After discarding the supernatant, 600 µl of Nuclei lysis solution was added and mixed via pipetting. The solution was then incubated at 80°C for five minutes and then cooled to room temperature. Once cooled, 3 µl of RNase A was added to the cell lysate and inverted 2-5 times to mix and incubated at 37°C for 60 minutes. 200 µl of protein precipitation solution was added and vortexed and then incubated on ice for five minutes. The solution was centrifuged at 19000 rpm for three minutes and the supernatant was transferred to a new centrifuge tube. 500 µl of Phenol Chloroform was added and mixed vigorously until a milky white color was observed. The solution was centrifuged for eight minutes and 30 seconds at 19000 rpm and the resulting top layer was added to a new centrifuge tube.

600 µl of isopropanol was added and shaken. The tube was inverted until a DNA pellet was visible in the solution. The tube was then centrifuged for three minutes at 19000 rpm at room temperature and the supernatant was discarded and drained on a Kim Wipe. 600 µl of EtOH was added and shaken until the pellet was suspended. The tube was centrifuged once more for three minutes at 19000 rpm and the supernatant was discarded. The resulting DNA pellet was air dried in the hood. Once the ethanol had fully evaporated, 50 µl of nuclease free water was added to the pellet and stored at 4°C (Newberry *et al.*, 2020). Total genomic DNA was subjected to amplicon sequencing via Illumina MiSeq with V3-V4 primers for taxonomic profiling of bacterial entities in the phyllosphere. Amplicon sequences of the v3-v4 region were assembled and filtered according to mothur's MiSeq SOP (http://www.mothur.org/wiki/MiSeq_SOP) with sequence

alignment to silva version 138. First the sequences needed to be denoised so the following commands were utilized: Make. contigs will look at each pair, take the reverse complement reverse read, and then determine the overlap between the two sequences. Our region of interest, the v3-vMake.contigs step, so the data is cleaned up from the problematic reads using screen.seqs which removes ambiguous bases and contigs longer than determined threshold. Microbiome samples usually contain large numbers of the same organism, and therefore we would expect to find many identical sequences in our dataset. Count.seqs is then used to combine the group file and the names file into a single count table. The next part of the pipeline in the align the sequences to our reference which is done by align.seqs. These alignments are further cleaned up using screen.seqs which will remove any overhang on either end of the V4 region and filter.seqs which will remove any columns that have a gap character. The near-identical sequences are grouped together with pre.cluster (these are Sequences that only differ by one or two bases). Now that the data has been cleaned up as much as possible, we now proceed with chimera filtering During PCR amplification *chimera* formation is possible, so we want to remove those sequences by using the chimera.vsearch command and remove.seqs will remove the sequences from the fasta file. Once the data is thoroughly cleaned, we can assign a taxonomy to the sequences using classify.seqs. The remove.lineage is also used here and will remove any 18S rRNA gene fragments or 16S rRNA from Archaea, chloroplasts, and mitochondria that have survived all the cleaning steps up to this point. Finally in cluster.split, sequences are split into bins, and then clustered with each bin based on the Taxonomic information. Mother merge produced a phyloseq object to be assessed in R studio. for the subsequent diversity analyses. Diversity analyses were assessed using the "vegan" package on R Studio. Principle component analyses (PCA) were conducted using the "microbiome" package using Aitchison distance.

Permutational multivariate analysis of variance (ADONIS) and Non-metric multidimensional scaling (NMDS) and based on Bray-Curtis dissimilarities were used to compare beta-diversity between treatments. The dispersion of the treatments was assessed with the function "betadisper" and compared to the individual samples using the "permutest" function.

Bacteriocin/Antibiotic Assays

To test for bacteriocin activity of *Xanthomonas perforans*, sterile glass plates were prepared with Nutrient Agar in which 10 µl of wild-type *Xanthomonas perforans* 10^8 cfu/ml inoculum was drop spotted onto the center of the agar. The plates were incubated at 28°C for about 48 hours. The plates were inverted, and 2-3 mL of Chloroform was added to the lid and left to evaporate for several hours until dry. The plates were then aerated in the biosafety cabinet for one hour. 0.3% water agar was prepared, and 3.5 mL was aliquoted into sterile test tubes. The phyllosphere strains were grown the over the previous evening and were prepared in 1 mL of MgSO4 in a centrifuge tube to make about 0.3 OD of inoculum. 200 µl of inoculum for each strain was added to individual tubes of water agar and vortexed. The water agar was then overlayed on top of the aerated plates. The glass plates were parafilmed and incubated for 24-48 hours. The procedure above was repeated with phyllosphere strains that were antagonistic to *Xanthomonas perforans* to test whether bacteriocins produced by the phyllosphere residents were inhibiting to *Xanthomonas perforans.*

Results

Phyllosphere Library Construction

The phyllosphere library constructed from the phyllosphere of tomato plants consists of 128 isolates representing approximately 20 genera including *Pseudomonas, Bacillus, Enterobacter, Pantoea, Stenotrophomonas, Staphylococcus, Ralstonia, Taonella, Rhizobium, Cupriavidus, Digitaria, Klebsiella, Leclercia, Delftia, Citrobacter, Burkholderia, Xanthomonas, Lysinibacillus, Exiguobacterium,* and *Chryseobacterium*. A majority of these representative strains belong to phylum Proteobacteria.

Contact-Dependent Competition Assays with Bacterial Phyllosphere Isolates

Through qualitative observation of the contact-dependent competition assays, it was determined that three phyllosphere isolates were inhibited by *Xanthomonas perforans* (Table 2-1). The inhibited strains included *Pantoea agglomerans, Enterobacter cloacae, and Stenotrophomonas maltophilia*. Contrarily, inhibition of *Xanthomonas perforans* by the phyllosphere isolates was observed with 17 strains. These strains include *Pseudomonas mosselii, Bacillus thuringiensis, Bacillus cereus, Pantoea agglomerans, Pseudomonas aeruginosa, Digitaria exilis, Stenotrophomonas maltophilia, Enterobacter ludwigii, Leclercia adecarboxylata,* and *Bacillus cereus.*

Bacteriocin Assays of inhibitor isolates

Bacteriocin assays of phyllosphere isolates causing inhibition of *Xanthomonas* (phyllosphere inhibitor) and of isolates that are inhibited by *Xanthomonas* (pathogen inhibitors) were conducted. The isolates that were inhibited by *Xanthomonas* displayed no inhibition from *Xanthomonas* when tested for bacteriocin activity for *Enterobacter cloacae* and *Pantoea agglomerans*. The isolates that inhibited *Xanthomonas* displayed zones of inhibition against

Xanthomonas growth for *Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Stenotrophomonas maltophila* (Figure 2-16).

Effect of wild-type and *tssM* mutant treatments on OTU richness and diversity

To further our insight on the role of T6SS in the phyllosphere, we used amplicon sequencing to study the influence of functional *tssM* (and thereby functional T6SS) on richness and diversity of the phyllosphere microbiome. The observed operational taxonomic unit (OTU) richness between treatments at the early timepoint were not significantly affected (P = 0.9553) (Figure 2-1). As time progressed, the OTU richness was also not significantly affected between treatments at the late timepoint (P = 0.7266) (Figure 2-1). The Inverse Simpson index was observed to be insignificant at the early timepoint (P = 0.2996) and the late timepoint (P = 0.1184) (Figure 2-2). The Shannon index was also observed to be insignificant at the early timepoint (P = 0.9101) and the late timepoint (P = 0.8357) (Figure 2-3). Comparison of the wild-type and *tssM* mutant treatments were evaluated as statistically insignificant at both timepoints for Observed (P= 0.8157, 0.5239), Shannon (P= 0.7276, 0.5707), and Inverse Simpson (0.7095, 0.09959) indices.

Effect of wild-type and *tssM* mutant treatments on phyla taxonomic composition and community structure

Phyla relative abundance across treatments show a similar trend throughout the early and late sampling timepoints. In both early and late timepoints, Proteobacteria is the most abundant phylum present in each treatment (Figure 2-4). The *tssM* mutant treatment consisted of low abundance of bacterial members in Bacteroidetes and Acidobacteria in the early timepoint and shifts in compositional structure to include Firmicutes, Bacteroidetes, Actinobacteria, and

Acidobacteria (Figure 2-4). The control/ uninoculated treatments show a similar composition of phyla between the two timepoints of Acidobacteria, Bacteroidetes, and Firmicutes, with an exceptionally reduced abundance of Firmicutes by the late timepoint (Figure 2-4). The phyla composition of the wild-type treatments is similar to the uninoculated treatments and remain consistent from the early to the late timepoint with a much higher abundance of Bacteroidetes (Figure 2-4). Prevalence filtering reveals Proteobacteria as the most dominant phylum throughout the treatments followed by Bacteroidetes, Actinobacteria, Firmicutes, and Acidobacteria (Figure 2-9).

Effect of wild-type and *tssM* mutant treatments on genera taxonomic composition and community structure

Evaluation of the top ten genera across treatments are consistent with varying levels of abundance (Figure 2-10). These genera include *Novosphingobium, Pantoea, Pseudomonas, Sphingomonas, Stenotrophomonas,* and *Xanthomonas*. Classification of OTU sequences placed some sequences to a certain threshold of identification including Burkholderiales, Enterobacteriaceae, Gammaproteobacteria, and Oxalobacteraceae. When comparing the wild-type samples between timepoints we see an increase of unclassified members of Gammaproteobacteria, *Oxalobacteracaeae*, *Stenotrophomonas*, and *Xanthomonas* while we see a decrease of unclassified members of Enterobacteriaceae, *Novosphingobium*, *Pantoea*, *Pseudomonas*. When comparing the *tssM* mutant samples between timepoints we see an increase of unclassified members of Gammaproteobacteria, *Oxalobacteracaeae, and Xanthomonas* while we see a decrease of unclassified members of Enterobacteriaceae, *Novosphingobium, Pantoea, Pseudomonas, Sphingomonas,* and *Stenotrophomonas.* At the early timepoint, we see that

members of Burkholderiales, Gammaproteobacteria and Oxalobacteraceae, *Novosphingobium*, and *Sphingomonas* are higher in abundance for the wild-type treatments compared to the *tssM* mutant treatments. Contrarily, we see that *Pseudomonas*, *Stenotrophomonas*, and members of Enterobacteriaceae are more abundant in the *tssM* treatments. By the late timepoint, members of Enterobacteriaceae and Oxalobacteriaceae, *Pantoea*, *Sphingomonas*, and *Stenotrophomonas* are more abundant in the wild-type treatments. The *tssM* treatments have a higher abundance of *Novosphingobium* and *Xanthomonas* at the late timepoint.

Beta diversity analyses

Principle component analysis (PCA) based on Aitchison distance was applied to our microbiome data to determine variance and separation between treatments. The first principle component (PC1) reveals 22.8% of the total variance and the second component (PC2) reveals 13.3% of the total variance for the early sampling. These components display a separation of the *tssM* mutant (red points, Figure 2-11) and wild-type samples (blue points, Figure 2-11) with exception to one *tssM* outlier. The late sampling's first principle component (PC1) reveals 22.8% of the total variance and the second component (PC2) reveals 11.9% of the total variance. These components do not display a separation between treatments (Figure 2-12).

Discussion

A comprehensive understanding of interactions between the phyllosphere microbiome and a plant pathogen allows for further application and the establishment of a healthy plant microbiome to suppress opportunistic plant pathogens. The influence of the Type VI Secretion System (T6SS) on the plant microbiome has been a gap in recent literature and is assessed in this study. The overall goal is to characterize phyllosphere microbial taxa associated with tomato and

how *Xanthomonas* interacts with them and alters the community structure during infection. Since *Xanthomonas* is equipped with T6SS, we have explored a question of whether the presence of T6SS allows *Xanthomonas* to alter phyllosphere microbial community structure. Further screening of the phyllophere library against the wild-type and *tssM* mutant strain provided insight of the influence of T6SS on varying genera and was verified through independent bacteriocin assays to determine whether T6SS is the initiator of inhibition.

In the analysis of the OTU alpha diversity, there were not significant differences between treatments in the Observed, Inverse Simpson, and Shannon diversity. While overall compositional diversity remains consistent between treatments, the relative abundance of phyla and key genera are observed to shift in comparison to treatment and time of sampling. Across all treatments, prevalence filtering reveals Proteobacteria with the most abundant presence followed by Bacteroidetes, Actinobacteria, Firmicutes, and Acidobacteria. In the early sampling, the *tssM* mutant treatment is predominantly Proteobacteria with a small presence of Acidobacteria and Bacteroidetes. The predominance of Proteobacteria is observed in both culture-independent and culture-dependent methods. The domination of Proteobacteria in the tomato phyllosphere has also been previously observed providing further verification of the role and presence of Proteobacteria in the phyllosphere (Dong *et al.* 2019 and Ottesen *et al.* 2013). Assessment of our microbiome data's beta diversity utilizing principle components analysis (PCA), reveals that the tssM mutant has a shift on the microbiome at day 7 post inoculation. Contrarily, PCA reveals that the tssM mutant and wild-type microbiomes converge to a similar composition by day 14 post inoculation. This data emphasizes the important of pathogen colonization and T6SS of X. perforans' ability to manipulate the microbiome composition.

Evaluation of genus composition classifies *Novosphingobium*, *Pantoea*, *Pseudomonas*, *Sphingomonas*, *Strenotrophomonas*, *Xanthomonas*, as well as unclassified members of *Burkholderiales* order, unclassified members of Enterobacteriaceae, Gammaproteobacteria, Oxalobacteraceae families as the top ten genera throughout all treatments. Further evaluation of individual relative abundance of genera reveals that members of Enterobacteriaceae decrease dramatically in the t*ssM* mutant treatment from the early to the late timepoint indicating a negative correlation that is not dependent on the T6SS. Members of Enterobacteriaceae were also seen to decrease slightly in abundance in the wild-type treatment, yet not as drastically as the *tssM* mutant treatment in the culture-independent approach. Comparison with our culture-dependent approach, the contact-dependent competition assays of *Enterobacter cloacae* against *X. perforans* reveals contradicting interactions with strains of varying identity. For example, *Enterobacter cloacae* (Ph. 31) has a query coverage of 97% and a percent identity of 100%. Ph. 31 is shown to be inhibited by wild-type *Xanthomonas* and does not show any inhibition in the bacteriocin assay indicating T6SS influence. *Enterobacter cloacae* (Ph. 95) has a query coverage of 99% and a percent identity of 99.03%. In contrast, Ph. 95 is shown to inhibit wild-type *Xanthomonas* and exhibits a zone of inhibition against *Xanthomonas* in the bacteriocin assay indicating the influence of bacteriocins. Another genus of interest, *Pantoea,* is seen to be relatively consistent between the *tssM* mutant and wild-type treatment and slightly higher in abundance in the inoculated treatments at the early timepoint. However, by the late timepoint, *Pantoea* is significantly reduced in the *tssM* mutant treatment compared to the wild-type treatment indicating a more aggressive relationship between *Xanthomonas perforans* and *Pantoea* in the absence of the T6SS in the culture-independent approach. Comparison with our culture-dependent approach, the contact-dependent competition assays of *Pantoea agglomerans*

against *X. perforans* also reveals contradicting interactions with strains of varying identity. For example, *Pantoea agglomerans* (Ph. 40) has a query coverage of 97% and a percent identity of 97.52%. *Pantoea agglomerans* (Ph. 40) is shown to be inhibited by wild-type *Xanthomonas* and does not show any inhibition in the bacteriocin assay indicating T6SS influence. On the other hand, *Pantoea agglomerans* (Ph. 8) has a query coverage of 65% and a percent identity of 97.95% and is observed to inhibit both the wild-type and *tssM* mutant strains, therefore further indicating possible genomic variability influencing these interactions. Our third genus of interest, *Stenotrophomonas* is shown to be more abundance in two of the *tssM* mutant samples at the early timepoint, however is shown to be significantly reduced across treatments and both timepoints with exception to one outlier in the wild-type for our culture-independent approach. Comparison with our culture-dependent approach, *Stenotrophomonas maltophilia* also displays contradicting interactions between two collected isolates further providing evidence on the importance of genomic variation within the phyllosphere community. *Stenotrophomonas maltophilia* (Ph. 22) has a query coverage of 84% and a percent identity of 96.70%. *Stenotrophomonas maltophilia* (Ph. 22) was shown to inhibit both the wild-type and *tssM* mutant strains. *Stenotrophomonas maltophilia* (Ph. 94) has a query coverage of 88% and a percent identity of 88.12% and contrarily is inhibited by the wild-type *Xanthomonas* and is observed to have a neutral interaction with the *tssM* mutant. Another intriguing genus, *Sphingomonas,* is shown to be significantly more abundant in the wild-type treatment in both early and late timepoints. This abundance may be correlated to promotion of inter-bacterial interactions in the presence of the T6SS. Data of *Pantoea* and *Sphingomonas* has suggested the possibility of diverse functionality for the T6SS other than pathogenicity and has been hypothesized to also mediate inter-bacterial (cooperative and communalistic) and bacteria-eukaryotic interactions (Jani and Cotter 2010).

This hypothesis of varying functionality correlates with the higher abundance of genera and observed aggressive behavior with the *tssM* mutant in both inter-bacterial interactions and disease severity. The variation of interaction between genera serves as an indicator of the dualistic functionality between mediation and pathogenicity.

Further observation of *Xanthomonas* at the early timepoint, shows the wild-type treatment to have a higher abundance in comparison to the *tssM* mutant. However, this completely shifts by the late timepoint in which *Xanthomonas* is observed to be more abundant in the *tssM* mutant treatment, while significantly reducing in the wild-type treatment. This shift can be attributed to two possible factors in which the wild-type *Xanthomonas* has successfully invaded the apoplast, therefore showing a reduced abundance on the phyllosphere or there is possible reproductive tradeoff in which the *tssM* mutant is able to produce more rapidly and abundantly by placing energy into reproduction rather than the expression and utilization of the T6SS. Demonstration of higher epiphytic fitness and disease severity has been previously observed by the *tssM* gene of T6SS-cluster i3* by Liyanapathiranage *et al.* (2021). Data presented here supports this understanding as we observe an increase in *Xanthomonas* in the *tssM* mutant of the greenhouse trials compared to the wild-type. Consideration of variable functionality and delivery of effectors to specific genera may explain the T6SS's role in cooperative and communalistic inter-bacterial interactions (Jani and Cotter 2010). One role of cooperative interactions stemming from T6SS has been suggested that the type VI effector proteins may play a role in the delivery of signals (Russell *et al.* 2014). A gut pathogen with a T6SS, *Campylobacter jejuni*, was observed to coexist with other microbes of the gut microbiome and was suggested that there is a cost of predation under environmental stress (Gupta *et al.* 2021). This correlates with our pathogen in which the environmental stress of the phyllosphere

may be a reason for mediation of T6SS utilization in these conditions. Another explanation for higher abundance of *Xanthomonas* mutant could be through reproductive trade off in which energy to contract and fire would be used in contrast to reproductive output. This hypothesis for trade-off of the T6SS has been previously identified for costs of reproductive rate and efficiency in proportion to the firing rate (Smith *et al.* 2020). Microbiome manipulation has been observed within the human gut in the presence of T6SS, however it has also been suggested that gut residents play a role in maintaining homeostasis in the presence of the antagonistic force (Allsopp *et al.* 2020). This correlates with the "gut health" of the plant in which the phyllosphere microbiome is shown to shift in the presence of T6SS during colonization, however our data shows the systems reach a consensus in homeostasis across the treatment samples. Overall, sampling size and higher inoculum concentrations could be expanded to determine the role of T6SS and decrease risk of variability. In conclusion, this study has revealed the importance of T6SS influence of the microbiome in the early colonization of *X. perforans* and dysbiosis among the pathogen and varying phyllophere residents.

References

Abrahamian, P., Klein-Gordon, J. M., Jones, J. B., & Vallad, G. E. (2021). Epidemiology, diversity, and management of bacterial spot of tomato caused by Xanthomonas perforans. Applied Microbiology and Biotechnology, 105(16), 6143-6158.

Allsopp, L. P., Bernal, P., Nolan, L. M., & Filloux, A. (2020). Causalities of war: the connection between type VI secretion system and microbiota. Cellular microbiology, 22(3), e13153.

Alvarez-Martinez, C. E., Sgro, G. G., Araujo, G. G., Paiva, M. R., Matsuyama, B. Y., Guzzo, C. R., ... & Farah, C. S. (2021). Secrete or perish: The role of secretion systems in Xanthomonas biology. Computational and Structural Biotechnology Journal, 19, 279-302.

Bernal, P., Llamas, M. A., & Filloux, A. (2018). Type VI secretion systems in plant associated bacteria. Environmental microbiology, 20(1), 1-15.

Bernal, P., Allsopp, L. P., Filloux, A., & Llamas, M. A. (2017). The Pseudomonas putida T6SS is a plant warden against phytopathogens. The ISME journal, 11(4), 972-987.

Boch, J., & Bonas, U. (2010). Xanthomonas AvrBs3 family-type III effectors: discovery and function. Annual review of phytopathology, 48, 419-436.

Choi, Y., Kim, N., Mannaa, M., Kim, H., Park, J., Jung, H., ... & Seo, Y. S. (2020). Characterization of type VI secretion system in Xanthomonas oryzae pv. oryzae and its role in virulence to rice. The plant pathology journal, 36(3), 289.

Dong, C. J., Wang, L. L., Li, Q., & Shang, Q. M. (2019). Bacterial communities in the rhizosphere, phyllosphere and endosphere of tomato plants. PloS one, 14(11), e0223847.

Gallegos-Monterrosa, R., & Coulthurst, S. J. (2021). The ecological impact of a bacterial weapon: microbial interactions and the Type VI secretion system. FEMS Microbiology Reviews, 45(6), fuab033.

Gupta, S., Ray, S., Khan, A., China, A., Das, D., & Mallick, A. I. (2021). The cost of bacterial predation via type VI secretion system leads to predator extinction under environmental stress. Iscience, 24(12), 103507.

Hachani, A., Lossi, N. S., & Filloux, A. (2013). A visual assay to monitor T6SS-mediated bacterial competition. Journal of visualized experiments: JoVE, (73).

Jacques, M. A., Arlat, M., Boulanger, A., Boureau, T., Carrère, S., Cesbron, S., ... & Vernière, C. (2016). Using ecology, physiology, and genomics to understand host specificity in Xanthomonas. Annual review of phytopathology, 54, 163-187.

Jani, A. J., & Cotter, P. A. (2010). Type VI secretion: not just for pathogenesis anymore. *Cell host & microbe*, *8*(1), 2-6.

Liu, H., Brettell, L. E., & Singh, B. (2020). Linking the phyllosphere microbiome to plant health. Trends in Plant Science, 25(9), 841-844. Trends in Plant Science, 25(9), 841-844.

Liyanapathiranage, P., Jones, J. B., & Potnis, N. (2021). A mutation of a single core gene, tssM, of Type VI secretion system of Xanthomonas perforans influences virulence, epiphytic survival and transmission during pathogenesis on tomato. Phytopathology, (ja).

Montenegro Benavides, N. A., Alvarez B, A., Arrieta-Ortiz, M. L., Rodriguez-R, L. M., Botero, D., Tabima, J. F., ... & Bernal, A. (2021). The type VI secretion system of Xanthomonas phaseoli pv. manihotis is involved in virulence and in vitro motility. BMC microbiology, 21(1), 1-12.

Ottesen, A. R., Gonzalez, A., Bell, R., Arce, C., Rideout, S., Allard, M., ... & Pettengill, J. B. (2013). Co-enriching microflora associated with culture based methods to detect Salmonella from tomato phyllosphere. PloS one, 8(9), e73079.

Russell, A. B., Peterson, S. B., & Mougous, J. D. (2014). Type VI secretion system effectors: poisons with a purpose. Nature reviews microbiology, 12(2), 137-148.

Schloss, P. D. (2020). Reintroducing mothur: 10 years later. Applied and Environmental Microbiology, 86(2), e02343-19.

Shyntum, D. Y., Nkomo, N. P., Shingange, N. L., Gricia, A. R., Bellieny-Rabelo, D., & Moleleki, L. N. (2019). The impact of type VI secretion system, bacteriocins and antibiotics on bacterial competition of Pectobacterium carotovorum subsp. brasiliense and the regulation of carbapenem biosynthesis by iron and the ferric-uptake regulator. Frontiers in microbiology, 2379.

Silverman, J. M., Brunet, Y. R., Cascales, E., & Mougous, J. D. (2012). Structure and regulation of the type VI secretion system. Annual review of microbiology, 66, 453-472.

Smith, W. P., Brodmann, M., Unterweger, D., Davit, Y., Comstock, L. E., Basler, M., & Foster, K. R. (2020). The evolution of tit-for-tat in bacteria via the type VI secretion system. *Nature communications*, *11*(1), 1-11.

Soria-Bustos, J., Ares, M. A., Gómez-Aldapa, C. A., González-y-Merchand, J. A., Girón, J. A., & De la Cruz, M. A. (2020). Two type VI secretion systems of Enterobacter cloacae are required for bacterial competition, cell adherence, and intestinal colonization. *Frontiers in microbiology*, 2137.

Thapa, S., & Prasanna, R. (2018). Prospecting the characteristics and significance of the phyllosphere microbiome. Annals of microbiology, 68(5), 229-245.

Zhang, Y., Callaway, E. M., Jones, J. B., & Wilson, M. (2009). Visualisation of hrp gene expression in Xanthomonas euvesicatoria in the tomato phyllosphere. European journal of plant pathology, 124(3), 379-390.

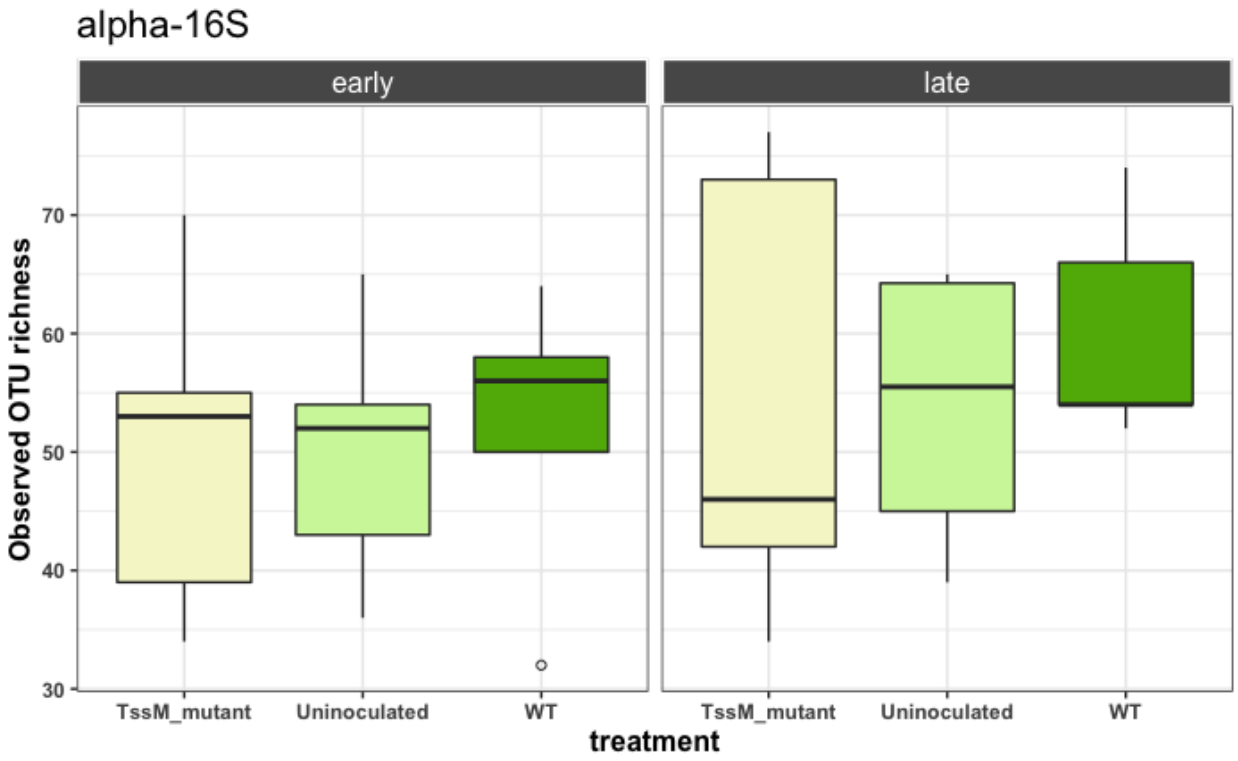


Figure 2-1: Observed OTU richness of phyllosphere treatments.

Observed OTU richness between *tssM* mutant, uninoculated, and wild-type treatments at early (7 days) and late (14 days) timepoints. Observed OTU richness between treatments at the early timepoint were not significantly affected (P = 0.9553). Observed OTU richness at the late timepoint was also not significantly affected between treatments (P = 0.7266).

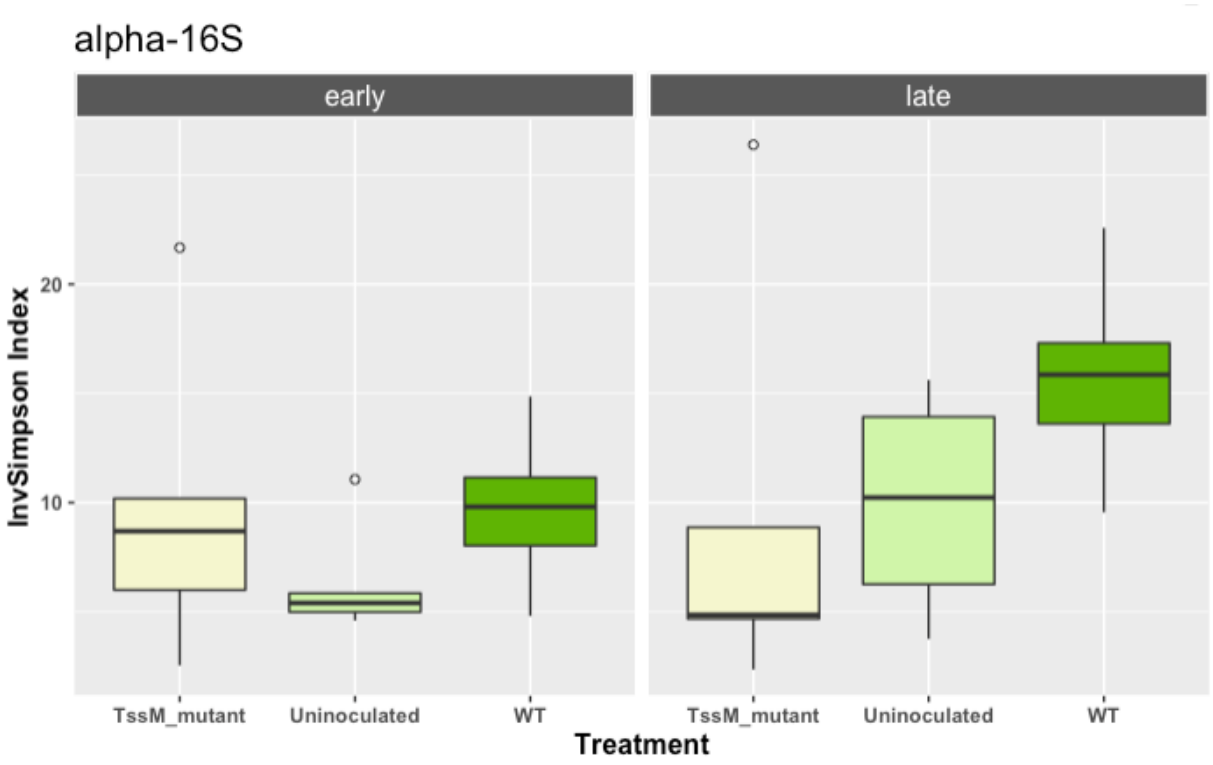


Figure 2-2: Inverse Simpson Index of phyllosphere treatments.

Inverse Simpson indices between *tssM* mutant, uninoculated, and wild-type treatments at early (7 days) and late (14 days) timepoints. The Inverse Simpson index was observed to be insignificant at the early timepoint (P = 0.2996) and the late timepoint (P = 0.1184).

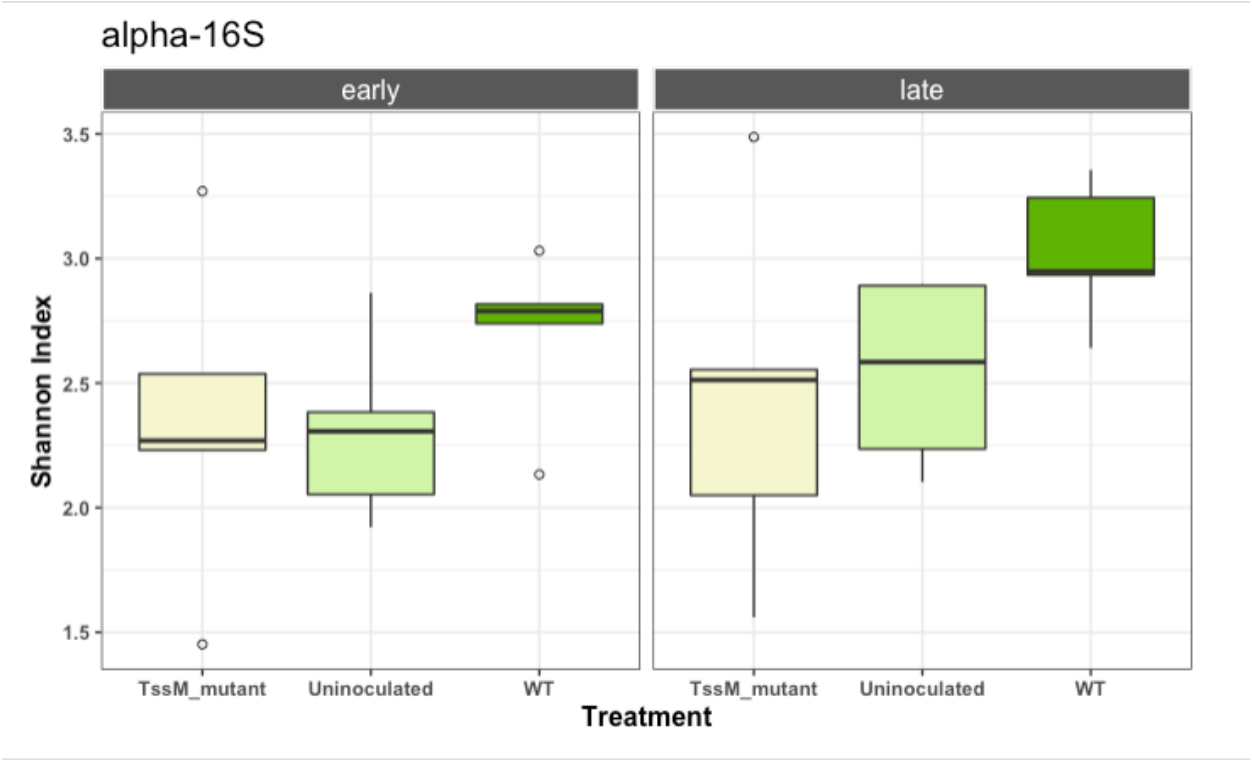


Figure 2-3: Shannon index of phyllosphere treatments.

Shannon indices between *tssM* mutant, uninoculated, and wild-type treatments at early (7 days) and late (14 days) timepoints. The Shannon index was observed to be insignificant at the early timepoint (P = 0.9101) and the late timepoint (P = 0.8357).

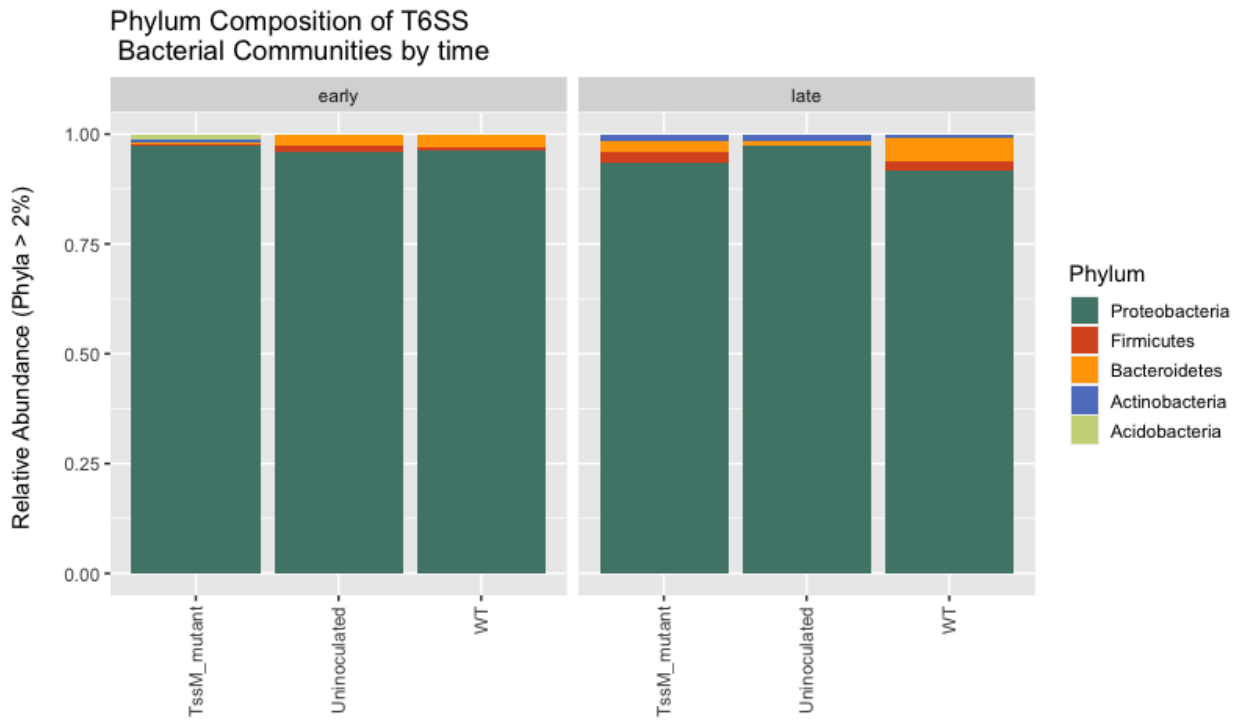


Figure 2-4: Phyla relative abundance of phyllosphere treatments.

Bar plots represent relative abundance of phyla for *tssM* mutant, uninoculated, and wild-type treatments at early (7 days) and late (14 days) timepoints. Individual bars display the average composition of individual treatments consisting on five tomato plant samples. The phyllosphere composition was surveyed from extracted DNA from the surfaces of the leaf tissues.

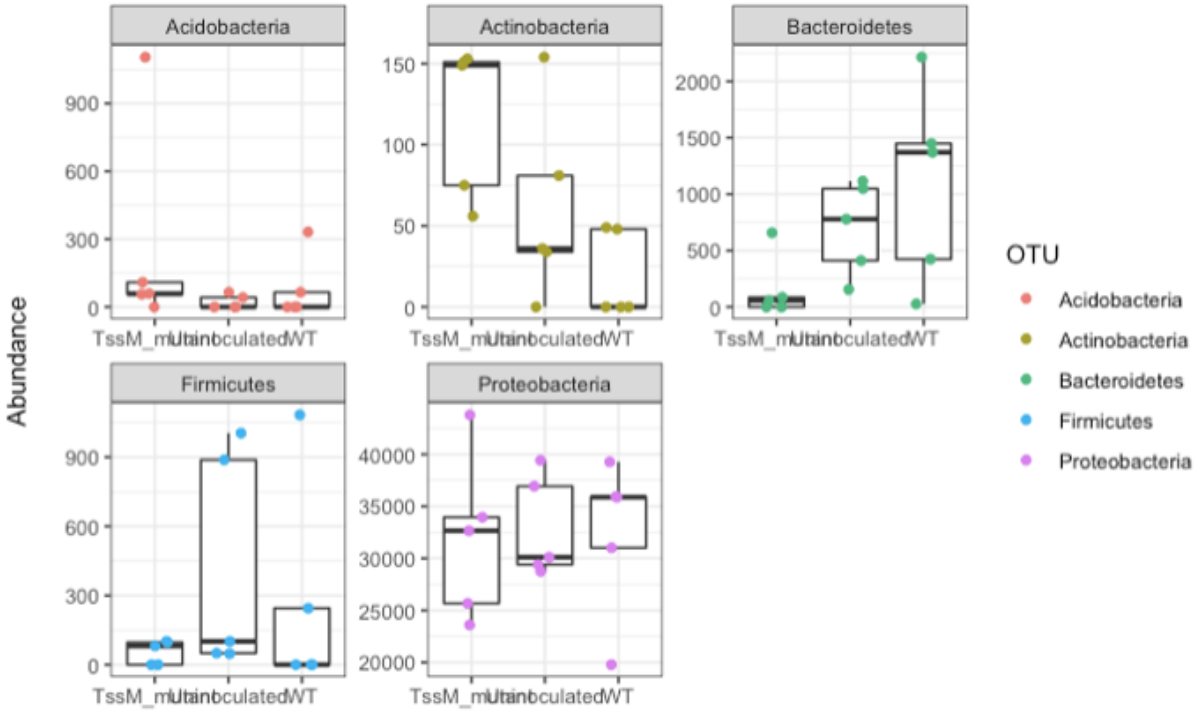


Figure 2-5: OTU phyla relative abundance for individual samples at early sampling.

Box and whisker plots displaying relative abundance of OTUs for individual phyla. Each phyla plot displays the relative abundance of phyla found in each individual five samples at the early sampling (day 7). Data points represent individual tomato plants. Black horizonal lines of the box plots display the average relative abundance between the five samples within a treatment. Whiskers display outlying samples.

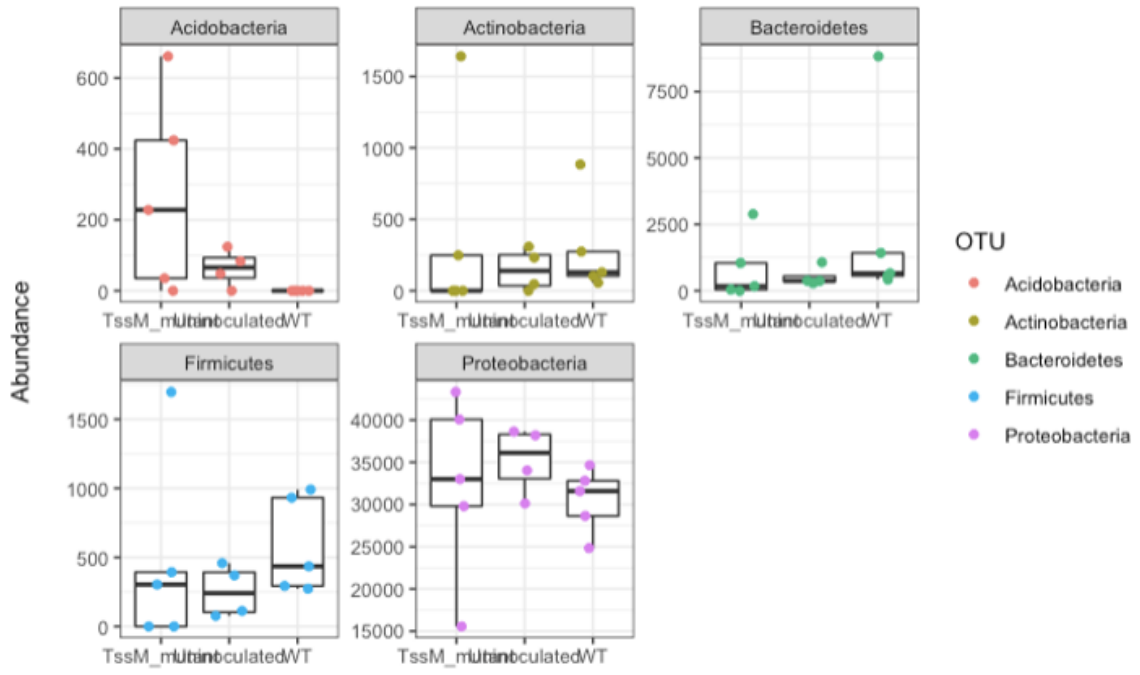


Figure 2-6: OTU phyla relative abundance for individual samples at late sampling.

Box and whisker plots displaying relative abundance of OTUs for individual phyla. Each phyla plot displays the relative abundance of phyla found in each individual five samples at the late sampling (day 14). Data points represent individual tomato plants. Black horizonal lines of the box plots display the average relative abundance between the five samples within a treatment. Whiskers display outlying samples.

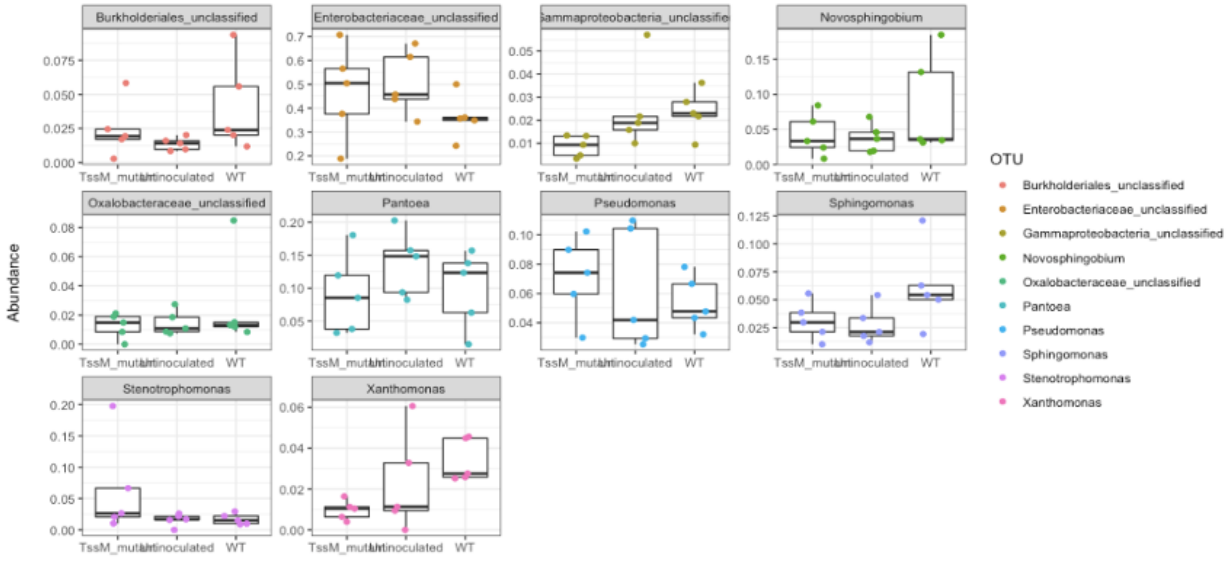


Figure 2-7: Top ten genera OTU relative abundance for individual samples at early sampling.

Box and whisker plots displaying relative abundance of OTUs for individual genera (top ten). Each genera plot displays the relative abundance of the top ten genera found in each individual five samples at the early sampling (day 7). Data points represent individual tomato plants. Black horizonal lines of the box plots display the average relative abundance between the five samples within a treatment. Whiskers display outlying samples.

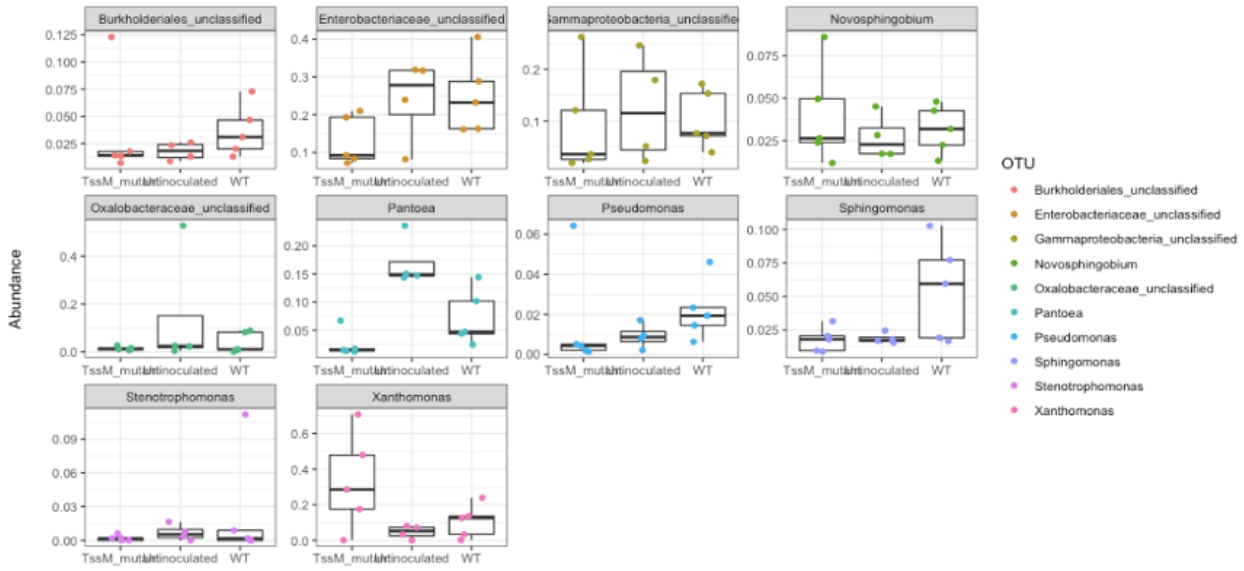


Figure 2-8: Top ten genera OTU relative abundance for individual samples at late sampling.

Box and whisker plots displaying relative abundance of OTUs for individual phyla. Each phyla plot displays the relative abundance of phyla found in each individual five samples at the early sampling (day 7). Data points represent individual tomato plants. Black horizonal lines of the box plots display the average relative abundance between the five samples within a treatment. Whiskers display outlying samples.

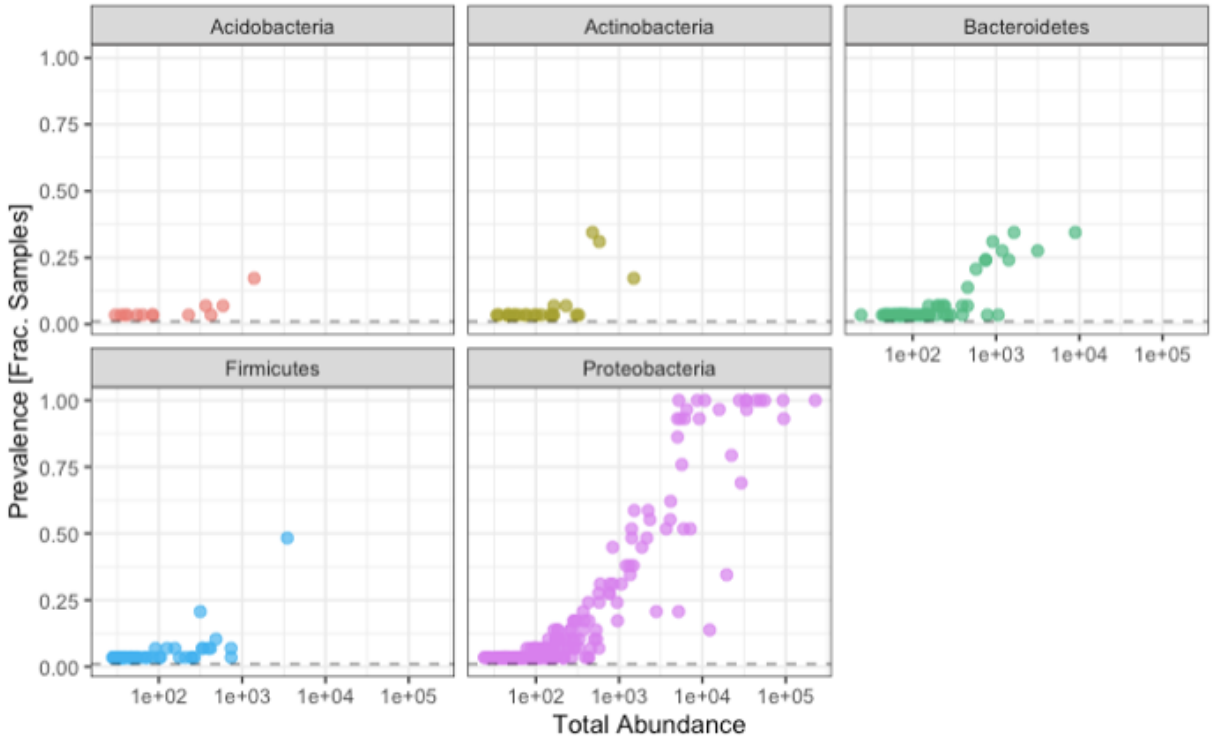


Figure 2-9: Prevalence Filtering across all combined treatments and sampling timepoints.

Prevalence plot of 1% (presence versus total count) for all combined treatments at the phylum level (prevalenceThreshold = 0.01). Data points represent different/unique taxa, while the y-axis represents the fraction of samples.

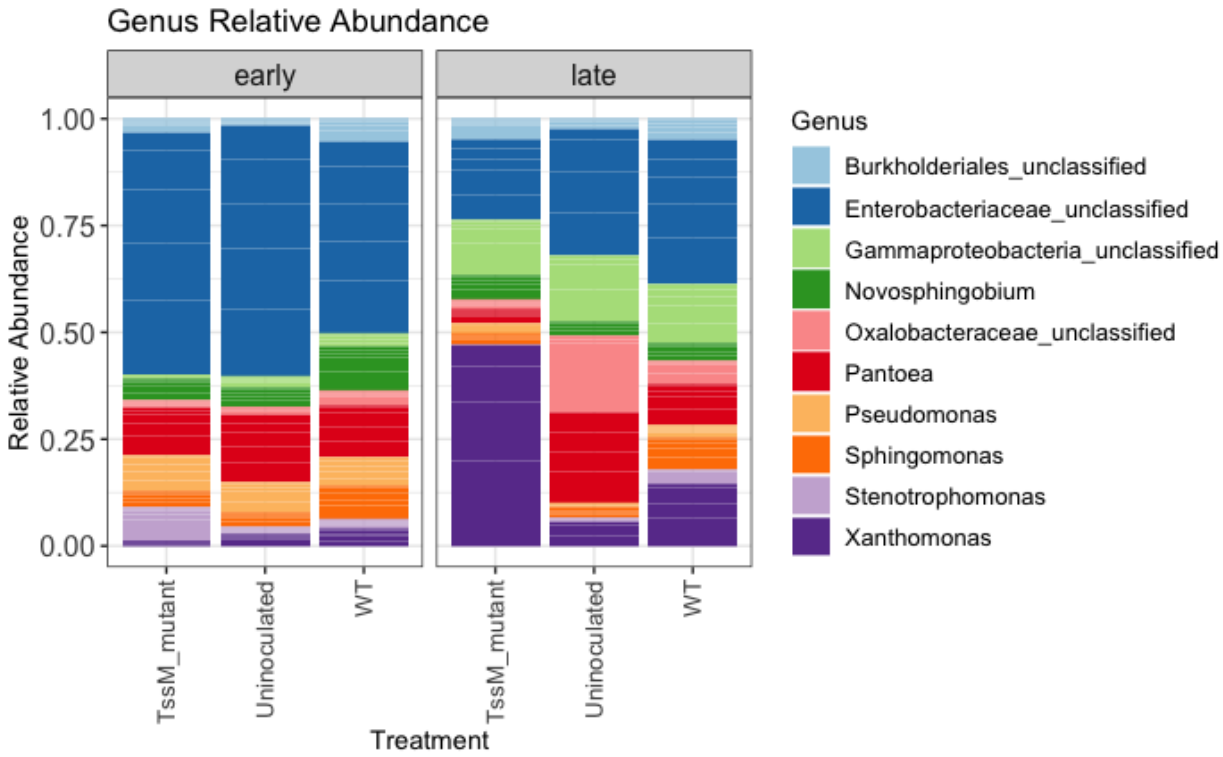


Figure 2-10: Top 10 genera and their relative abundance within each treatment at both early and late timepoints.

Bar plots represent relative abundance of the top ten genera for *tssM* mutant, uninoculated, and wild-type treatments at early (7 days) and late (14 days) timepoints. Individual bars display the average composition of individual treatments consisting of five tomato plant samples. The phyllosphere composition was surveyed from extracted DNA from the surfaces of the leaf tissues. Taxa that could not be assigned to a genus was reported at the next specific classification.

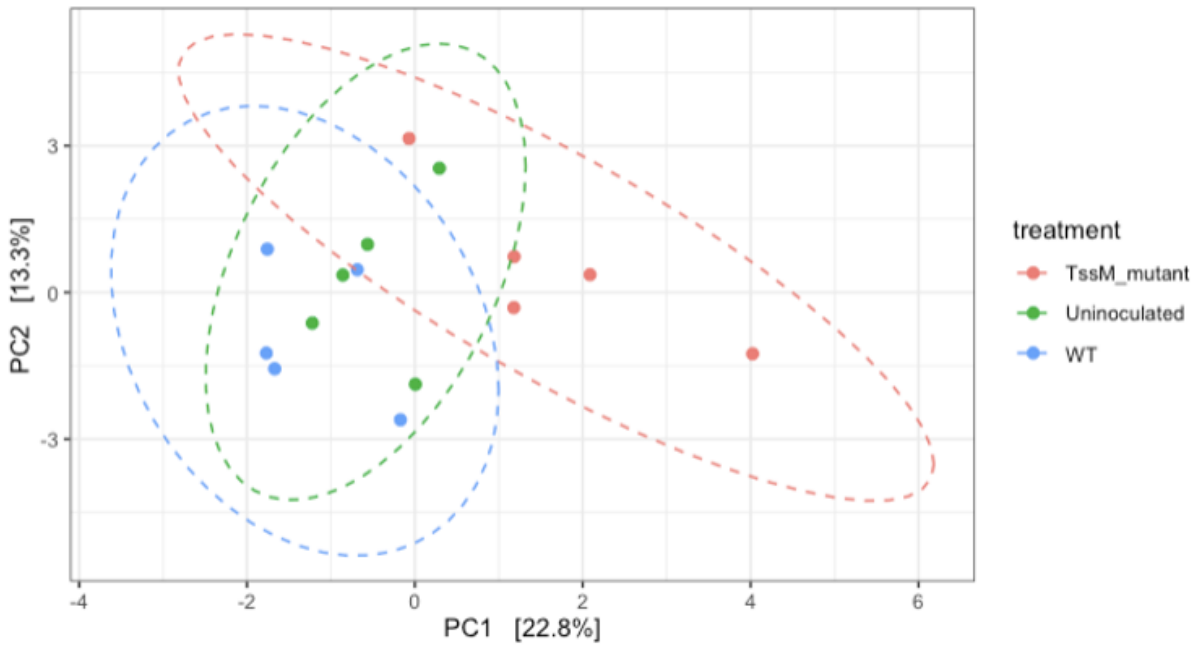


Figure 2-11: Principle components analysis (PCA) of early sampling.

Beta diversity ordination based on Aitchison distance at the early sampling (day 7). Principle components analysis (PCA) was applied to the centered-log ratio transformed counts using the microbiome package in R. Treatment ellipses are differentiated by color. Data points represent individual tomato plant samples.

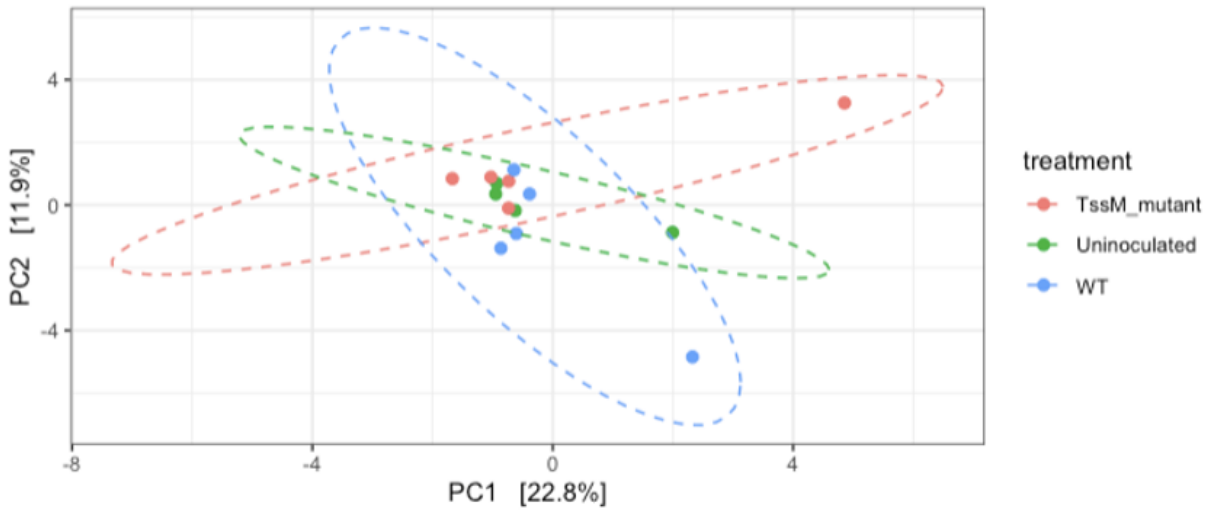


Figure 2-12: Principle components analysis (PCA) of late sampling.

Beta diversity ordination based on Aitchison distance at the late sampling (day 14). Principle components analysis (PCA) was applied to the centered-log ratio transformed counts using the microbiome package in R. Treatment ellipses are differentiated by color. Data points represent individual tomato plant samples.

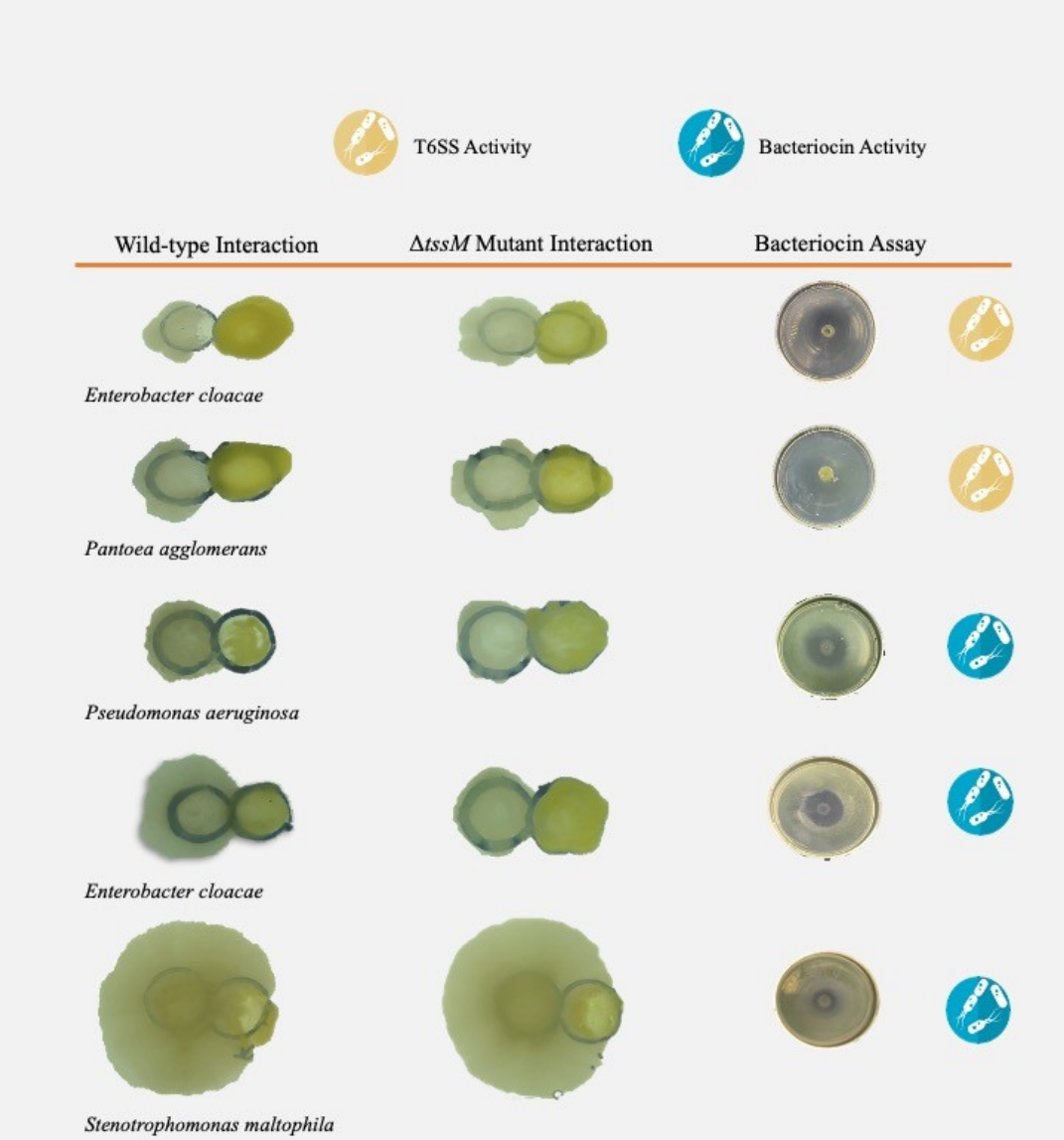
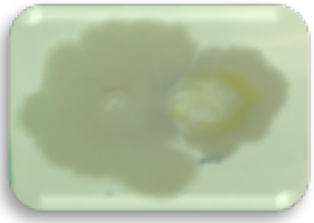
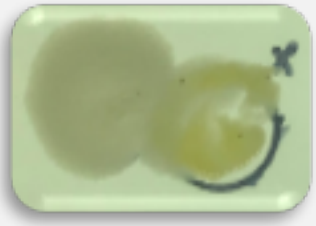
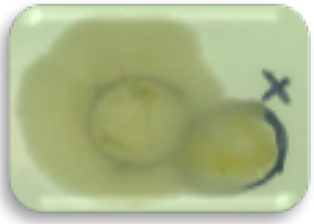
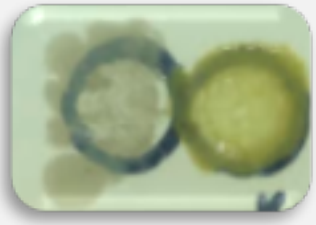
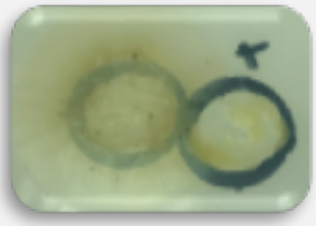
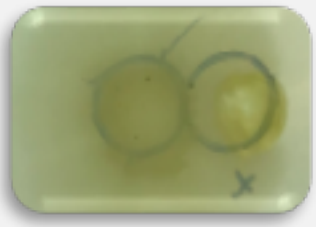
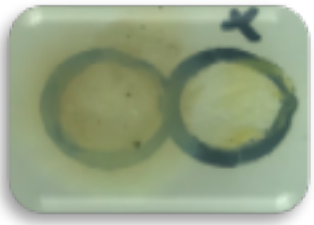
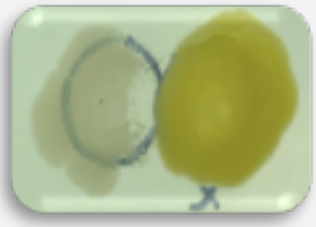
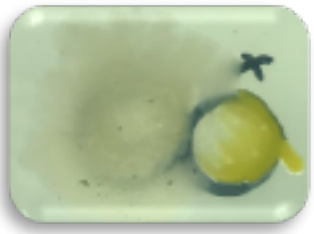
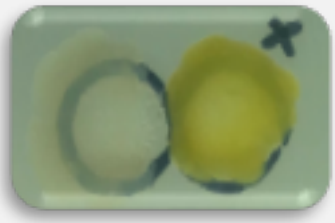
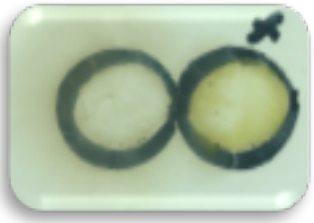
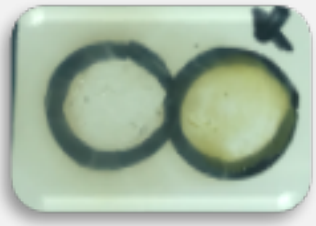
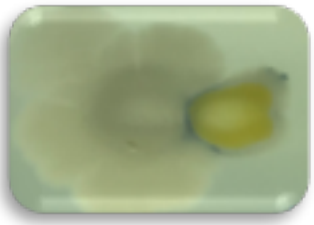


Figure 2-13: T6SS and bacteriocin interaction mediator assays.

Contact-dependent competition assays between wild-type and *tssM* mutant along with bacteriocin assays to determine T6SS or bacteriocin activity. First column displays representative interactions between *Xanthomonas perforans* wild-type and a phyllosphere isolate. Second column displays the interaction of the phyllosphere isolate against the *tssM* mutant of *X. perforans*. Third column displays bacteriocin assays between the phyllosphere isolate and *X. perforans*. Symbols to the right indicate T6SS (yellow) or bacteriocin (blue) activity.

	Strain Identity	Query Coverage	Percent Identity	Interaction	+/-/=
Ph 2	*Pseudomonas mosselii*	89%	99.48%		-
Ph 4	*Bacillus thuringiensis*	88%	99.65%		-
Ph 5	*Bacillus cereus*	66%	99.45%		-
Ph 8	*Pantoea agglomerans*	65%	97.95%		-
Ph 15	*Pantoea agglomerans*	99%	98.69%		+

Ph 19	*Pseudomonas aeruginosa*	88%	99.35%		-
Ph 20	*Pseudomonas aeruginosa*	98%	96.62%		-
Ph 21	*Digitaria exilis*	99%	98.99%		-
Ph 22	*Stenotrophomonas maltophilia*	84%	96.70%		-
Ph 23	*Enterobacter ludwigii*	95%	100.00%		-
Ph 31	*Enterobacter cloacae*	97%	100.00%		+

Ph 33	*Leclercia adecarboxylata*	92%	96.65%		-
Ph 40	*Pantoea agglomerans*	97%	97.52%		+
Ph 77	*Enterobacter ludwigii/ Kobei*	98%	97.57%		-
Ph 78	*Pseudomonas aeruginosa*	99%	99.11%		-
Ph 82	*Bacillus thuringiensis*	99%	99.14%		-
Ph 90	*Stenotrophomonas maltophilia*	93%	92.17%		-

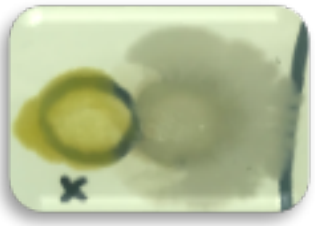
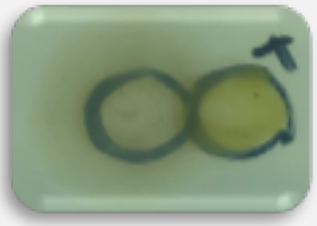
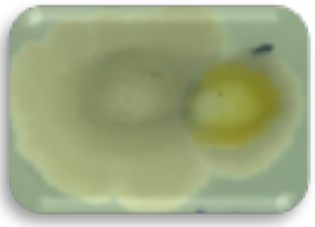
Ph 94	*Stenotrophomonas maltophilia*	88%	88.12%		+
Ph 95	*Enterobacter cloacae*	99%	99.03%		-
Ph 124	*Bacillus cereus*	99%	99.91%		-

Table 2-1: Contact- dependent competition assays

Contact-dependent competition assays between wild-type *Xanthomonas perforans* and phyllosphere isolates of the phyllosphere library showing negative interactions. (+) indicates *Xanthomonas* as the inhibitor. (-) indicates inhibition of *Xanthomonas* from the phyllosphere isolate. Query coverage and percent identity are included for 16S rDNA sequencing of phyllosphere isolates.

3. CHAPTER THREE

Assessment of Intraspecific Diversity's Influence on Disease Severity

Abstract

With the advancement of sequencing technologies, pathogen diversity has become evident and characterized in field samples, however consequences of this intraspecific diversity on overall disease outcomes have yet to be investigated for *Xanthomonas perforans*, the causal agent of bacterial leaf spot (BLS) of tomato. In previous studies conducted by our lab, we identified that *X. perforans* populations has recently diverged into at least six sequence clusters and more than single genotype can co-occur in the infected fields. In this study, we evaluated consequences of co-occurrence of multiple pathogen genotypes on overall disease severity under greenhouse conditions, where influence of environmental variation such as rainfall and humidity are minimal. Combinations of the genotypes identified at the field level were co-inoculated on tomato plants using dip-inoculation method and assessed for disease severity at varying time points. Our findings revealed a trend of higher disease severity in co-infections of two or more genotypes in comparison to infection by a single pathogen genotype.

Introduction

Plant disease can be measured in epidemiological terms and disease severity (Kranz 1988). Measurement of disease severity and consequential effect on crop yield is important to determine control and management. Disease severity data are essential to help predict disease progress and disease severity throughout the growing season. Disease severity also correlates to quality of crop yield/ product therefore influencing the value and profitability of the crop (Kranz

1988). Quantification of plant disease has traditionally been dominated through visualization and perception of symptomatic intensity which has therefore been subject to sources of human error (Chiang *et al.* 2021). Plant disease severity is typically defined as the area of a sampled unit expressing a proportion or percentage of symptomatic tissues (Chiang *et al.* 2021).

Bacterial leaf spot (BLS) of tomato caused by *Xanthomonas* is favored by high humidity and warm temperatures (Bhattarai 2016; Abrahamian *et al.* 2021). Symptoms of BLS present necrotic, water-soaked leaf spots surrounded by a yellow halo induced by chlorosis on leaves, stems, sepals, pedicels, and fruits (Lewis *et al.* 2016; Mbega *et al.* 2013; Potnis *et al.* 2015). Colonization of apoplast by *X. perforans* takes place upon entry through openings in the host plant such as stomata, lenticels, hydathodes, and wounds (Jacques *et al.* 2016). *X. perforans*, a causal agent of BLS has revealed an array of effectors which aide in the increase of fitness, virulence, and dissemination (Abrahamian *et al.* 2021).

In the previous study conducted in our lab, isolate genome analyses revealed that *Xanthomonas perforans* has recently undergone diversification on tomato into at least six sequence cluster or genotypes. The eight sequenced genomes were compared to previously published genomes from GenBank. These comparisons revealed the emergence of novel transcription activator-like effectors and independent recombination events with *X. euvesicatoria*. A maximum-likelihood phylogeny constructed from core genome single-nucleotide polymorphisms which revealed the presence of six sequence clusters (SC) within *X. perforans*. SC1-SC4 were shown to relate to previously described populations in Florida. SC3 includes a strain isolated in Florida from pepper named Xp2010 is used as a representative strain in this study for SC3. Pepper strains isolated in Alabama from pepper form a distinctive lineage known as SC6 including our representative strain AL65. A strain isolated in Alabama, AL57,

was observed to group within the cluster of SC4. Branching from SC4, two Alabama strains AL33 and AL37, composed SC5 in which AL37 is utilized as a representative from this cluster (Newberry *et al.* 2019). In field DNA collections, our previous data underwent two rounds of single nucleotide variant profiling using StrainEst which revealed the co-occurrence of the *X. perforans* SCs found in individual field samples.

In samples of varying fields across Alabama, the co-occurrence of *X. perforans* SCs was assessed and reported. In Macon County, an individual field revealed the presence of three co-occurring SCs: SC5, SC3, and SC6. The Lee County community garden revealed the presence of SC5 and SC6 co-occurring. Tuscaloosa County had two samples in which two co-occurring SCs were revealed. One sample included SC3 and SC6, while the other sample included co-occurrence of SC3 and SC5. The sample from DeKalb County revealed the co-occurrence of SC3 and SC4 (Newberry *et al.* 2020). These observations led us to hypothesize that the mixed infections of multiple genotypes can have different outcomes in terms of overall disease severity when compared to single infections. To address this hypothesis, we selected the genotype combinations identified to be prevalent in the recent field sampling, these include: SC 5/6, SC 3/5/6, SC 3/5, SC 3/6, and SC 4/3.

Materials and Methods

Greenhouse dip-inoculations to assess disease severity

To determine the effects of intraspecific diversity of *X. perforans*, 4–5-week-old tomato cv. FL47 plants were dip-inoculated (about 30 seconds each) in 600 mL cell suspensions containing

about 1 X 10^6 cfu/mL of representative strains of each genotype and co-infection mixtures that were previously observed in tomato fields across Alabama. Treatments consisted of a control, SC 6, SC 5/6, SC 3/5/6, SC 5, SC 3/5, SC 3, SC 3/6, SC 4, and SC 4/3. The control treatment only consisted of 0.01M $MgSO_4$ amended with 0.00025% of Swilvet. Five replicate tomato plants (FL47) about six weeks old were dip-inoculated of each treatment into the inoculum poured into sterilized beakers and spread apart in the greenhouse. High humidity treatments were placed in plastic bins with a plastic covering during the evening hours for the first week while low humidity remained in ambient conditions of the greenhouse. The plants were watered daily and assessed for disease severity on days 7 and 14 with additional ratings on Days 9 and 12 for plants with delayed disease development due to temperature fluctuations. The disease severity scale was based on the Horsfall-Barratt scale for the percentage of symptoms to plant tissue (Hollis 1984). No symptoms were rated as 0, 1-3% as 1, 3-6% as 2, 6-12% as 3, 12-25% as 4, 26-50% as 5, 51-75% as 6, 76-87% as 7, and 88-100% as 8. Sources of error in disease severity perception were attempted to be counteracted through the observations of two individuals in which the disease severity index were averaged. Raw disease severity indices were utilized for area under the disease progress curve (AUDPC), a method where multiple observations of disease progress are correlated to a single value. AUDPC is calculated as follows (Simko and Piepho 2012):

$$AUDPC = \sum_{i=1}^{n-1} \frac{y_i + y_{i+1}}{2} \times (t_{i+1} - t_i)$$

Population studies

On the seventh and fourteenth day of the four November greenhouse trials, about three leaves were sampled from each replicate in each treatment and placed in Ziplock bags for

transport from the greenhouse. Leaves were punched for four disks of lead tissue with a cork borer. The four leaf disks were placed in 1.5 mL centrifuge tubes with 200 µl of 0.1 $MgSO_4$. This process was repeated for each of the three replicates for each treatment in both low and high humidity conditions. The four leaf disks were then grinded with a Dremel until the tissue was fully macerated. After grinding, 700 µl of 0.1 $MgSO_4$ was added to each tube. Dilutions were made by taking 100 µl of the 10^0 stock and adding it to a centrifuge tube with 900 µl of 0.1 $MgSO_4$. These dilutions were repeated to a dilution factor of 10^{-5}. Each dilution factor was plated on Nutrient Agar and incubated for two to three days at 28ºC. Plates were counted for individual colonies and assessed for numbers between 30-300 colonies. Population for each replicate was calculated using the following formula:

$$\frac{\text{(Total number of colonies) X (Dilution factor)}}{\text{(Volume plated) X (Cork borer area)}}$$

Statistical analyses

AUDPC was plotted for each treatment across seven experimental batches using the package “dplyr" on R Studio (Wickham *et al.* 2014). The values obtained from the AUDPC were then utilized to build a linear model and linear effect mixed model with “lme4” and “lmerTest” (Bates *et al.* 2007; Kuznetsova *et al*. 2015). To see how individual strains compare to each other, analysis using a generalized linear hypothesis test, while adjusting for multiple comparisons using Tukey's method was utilized. To assess the difference in slopes, disease development was analyzed using linear mixed effect models.

Results

Area under disease progress curve (AUDPC)

In the first experimental batch, treatment 5/6 has the highest AUDPC value while treatment 6 has the lowest AUDPC value (Figure 3-3). The next highest AUDPC value is treatment 3/6 followed by treatment 5. The second experimental batch has 4/3 with the highest AUDPC and 5 as the lowest. Treatments 3/5 and 3/6 show similar AUDPC values to treatment 4/3. Two-genotype infections are shown to have a higher AUDPC trend compared to the single-genotype infections. In the third experimental batch, treatments 3/5, 4/3, and 5/6 have the highest AUDPC values while treatments 3, 4, and 6 have the lowest AUDPC values, indicating higher AUDPC values in the two-genotype infections compared to single-genotype infections. Treatment 4/3 has the highest AUDPC value in experimental batch four while treatment 6 has the lowest AUDPC value. The next highest AUDPC value is treatment 3/5/6 followed by treatment 3. In the fifth experimental batch, treatment 4/3 displays the highest AUDPC value, with treatment 4 as the lowest followed by treatment 5. Treatment 3 shows the greatest variation among treatments. Overall, the two-genotype infections show a higher AUDPC trend in comparison to the single-genotype infections in this batch. The sixth experimental batch, treatment five shows the highest AUDPC value by a slight margin and followed by treatments 3/5 and 3. The lowest AUDPC value is treatment 6 followed by treatment 5/6. In the seventh experimental batch, treatment 3/5/6 has the highest AUDPC while treatment 4 has the lowest. Across the experimental batches a trend is seen with co-infection of two of more genotypes having a significantly higher AUDPC values compared to single-genotype infections. In

summary, we observed a significant variation on the disease severity values and thus AUDPC values in different batches of experiments.

The AUDPC values were further assessed taking into account the batch effect and fit for linear model and mixed effect model to the data was evaluated. Mixed effect model was better fit for this dataset. Batch effects were determined to be significant (p-value < 2.2e-16). The post-hoc tests indicated significant p-values between SC3 and SC4 treatments as well as SC3 and SC 4/3 treatments (Figure 3-4). To observe how individual strains compare to each other, a generalized linear hypothesis test with adjusted multiple comparisons using Tukey was utilized and determined statistical significance in pairwise comparisons 4 and 3/5, 5 and 3/5, 6 and 3-5, 4 and 3/5/6, 5 and 3/5/6, 6 and 3/5/6, 4/3 and 4, and 6 and 4/3 (Figure 3-5). Coinfection with SC3 and SC5 showed significantly higher AUDPC values compared to single infections by SC4 (P= 0.01167), SC6 (P= 0. 04506), and SC5 (P= 0.04519), but not SC3 alone. AUDPC for coinfection by SC3 and 5 was not significantly different than by SC3 alone. Coinfection with SC3, SC5, and SC6 also displayed significantly higher AUDPC values compared to single infections by SC 4 (P= 0. 00820), SC5 (P= 0. 03225), and SC6 (P= 0. 03181). Coinfection with SC4 and SC3 showed significantly higher AUDPC values compared to the singles infections by SC4 (P= 5.70304e-5), SC5 (4.89449e-4), and SC6 (P= 5. 70366e-4).

Analysis of disease development

While AUDPC analyses had shown some treatments to have similar areas, there was differences in the slope indicating a distinguishable difference in disease development. Disease development was assessed using linear mixed effect models. The lmer model considers fixed effects from genotype/SC and a fixed interaction between strain and time with random effects of

batch. The following pairs show significant differences in disease development: SC4-SC4/3, SC5-6-SC4, SC4-SC3/5/6, SC4-SC3/6, SC6-SC4/3, SC5-SC3/5/6, SC5-4/3, SC3/5/6-SC3, and SC3/6-SC3. Disease development appears to be highest between 4-8 DPI in SC3/5, 3/5/6, 3/6, 4/3, and 5/6 across treatments (Figure 3-7).

Population studies

Population of *Xanthomonas perforans* genotypes among treatments remain relatively consistent among treatments at both time points (Figure 3-8). No difference in population levels was detected among treatments at either sampling according to Tukey's test of least significant difference.

Discussion

The objective of this study was to determine whether the presence of mixed infections with multiple genotypes of *Xanthomonas perforans* leads to higher or lower disease severity on the host and whether it influences overall disease development when compared to single infection. We observed that mixed infection by genotype combinations evaluated here results in higher disease severity compared to individual infections by each genotype, with exception of SC3. Write what was different with SC3 and mixed infection with SC356/34 etc. The significance of this study and its finding are relevant due to the increasing presence of mixed genotype infections across fields (Klein-Gordon *et al.* 2022; Newberry *et al.* 2019). Understanding of mixed infections leading to higher disease severity is necessary to mitigate prediction of severity within a field and management efforts within the production system to limit increasing genotypic variation (Klein-Gordon *et al.* 2022).

Mixed infections have been indicated to be more prevalent in the field in contrast to single infections (Newberry *et al.* 2019; Barrett *et al.* 2021; Mideo 2009). Most mixed infections are expected to report a higher severity output on the host plant (Stubbendieck and Straight 2016). Intraspecific diversity of necrotrophic plant pathogens has indicated variation of pathogenesis in previous studies of fungi (Rowe and Kliebenstein 2010). Co-infection studies with fungi reveal that co-infection with two strains of *Podosphaera plantaginis* on *Plantago lanceolata* result in higher disease prevalence, severity, and transmission rates (Susi *et al.* 2015). Similar findings have been reported in other hosts with the infection of Zebra fish (Danio rerio) with *Flavobacterium columnare* in which intraspecific competition between the strains has been attributed to the increase in virulence (Kinnula *et al.* 2017). This literature corresponds with our findings in that mixed infections of intraspecific genotypes lead to higher disease severity for a bacterial plant pathogen. Experimental exploration of the genotypes by tagging each strain with fluorescence/antibiotics is an avenue that we had wished to explore and could be used for future studies to determine the fitness of each individual genotype within a mixed infection.

Assessment of genotype-genotype interactions need to be further evaluated, however potential mechanisms influencing these interactions may include competitive advantages and trade-off of various virulence factors between strains. Competitive advantage between the strains with variable factors such as temperature, humidity, or genetic diversity, may allow for the favoring of specific strains. For example, versatile metabolism can provide a competitive advantage for some strains in which scavenging of scarce nutrients is assessable (Bulgarelli *et al.* 2013). After inoculation, advantages such as a more versatile metabolism may allow for specific strains to adapt to the phyllosphere conditions more rapidly compared to others, therefore allowing a more rapid and efficient invasion of the host's apoplast. Effectors are secreted

proteins that vary in functionality and may be required for pathogenicity of the pathogen (White *et al.* 2009). Effectors of *Xanthomonas* have been reported to be essential for pathogenicity, host specificity, and pathogen fitness (Alvarez-Martinez *et al.* 2021). When assessing the effectors within the individual sequence genotypes of *Xanthomonas perforans*, we can see variability in the presence of eight effectors out of 30 known effectors. It has been noted that most *X. perforans* strains carry a Type III secreted effector named AvrXv3 or AvrBsT. This effector has been observed to elicit effector-triggered immunity. Acquisition of AvrBsT has previously been attributed as a fitness advantage of *X. perforans* based on disease severity observations (Abrahamian *et al.* 2018). One of our previous studies also indicates that an insertion sequence is found within the encoding region of AvrXv3 in AL65 of SC6. The presence of AvrBsT however has only be identified in AL57 (SC4) and AL37 (SC5) as indicated in Table 3-1. Loss of this gene has been observed to cause a fitness penalty to the pathogen indicating its role in fitness of the co-infections present. The presence of XopAQ and XopE3 were only found to be present in AL57 of SC4 and Al37 of SC5, however XopE3 was interrupted in both strains by an insertion sequence (Newbery *et al.* 2019). These effectors have been found to be homologs which suppress plant immunity (Barak *et al.* 2016). XopAQ is also only found in AL57 (SC4) and AL37 (SC5). A novel transcription activation-like effector (TALE) previously designated as PthXp1which contributes to virulence and has been attributed to possibly causing a contribution to the emergence of SC4 and SC5 in the southeast United States as it is also seen to be present in our representative strains AL57 (SC4) and AL37 (SC5). XopE2 has been observed to be involved in virulence and suppression of hypersensitive response (Lin *et al.* 2011). However, this effector is only present in one of our representative strains, AL57 (SC4). Other TALE effectors, AvrHah1 and XopAO, has been associated with the advancement of disease development on

tomato (Newberry *et al.* 2019). These TALE effectors have only been associated with one representative strain in this study, AL65 (SC6). When associating the effectors with the given treatments, the largest indicator of effector influence is from treatment 5/6. This two-genotype infection remained relatively high across trials held in October and November. What is intriguing, is that both strains produce effectors not seen in the other indicating a possible trade-off between the two strains. When placed in a similar niche, the strains have full advantage for infection due to the interplaying capability of the strains. The three-genotype infection (3/5/6) show production of AvrXv3 in AL37 (SC5) along with a disrupting insertion sequence seen in AL65 (SC6) and an inactivated version in Xp2010 (SC3). The largest contributor to this co-infection could be SC5 followed by SC6 based on the production of varying effectors seen to be carried by these strains, however further evaluation of individual population within the treatment and effector production will need to be taken into consideration to establish this connection. Treatment 4/3 and 3/5 do show slightly more aggressive tendencies, however, is variable among treatments as well. These pairs may be well-suited for infection due to SC4 and SC5 having the ability to overcompensate effector production in contrast to SC3. Single infection of SC3 did not show significantly different AUDPC values in comparison to co-infection with SC3 and SC5 which may indicate that redundancy of effector output between strains may not be a sole influencer on disease severity. Significant contrasts in AUDPC values and disease development reveal a trend in higher disease progression of co-infections with two or more genotypes compared to single-genotype infections with an exception to variable treatments. The variation of Type III secreted effectors among these clusters have also been suggested to influence different fitness ability of the pathogen on the host (Klein-Gordon et al. 2022). Overall, effector production within individual genotypes may be the leading contributor to disease severity when

placed in mixed infections of a single niche. However, the output of effectors in this study is theoretical due to unknown expression of effectors within the treatments. Further evaluation of effector expression and genotype fitness may provide more substantial evidence behind the mediators of these interactions.

Mixed infections of plant viruses are known to be common in nature and provides a framework for the importance of co-infections across plant pathogens (Rochow 1972). Our data suggests that severity depends on the strains and combinations within a single niche. Viral mixed infections have been extensively study and reveal complex infections which have been shown to rely on the pathogen, host, and vector(s) and the interactions between them. Viral mixed infections studies have reported that viral combinations and their respective outcomes vary depending on the host cultivar (Moreno and López-Moya 2020). Mixed double or triple viral infections have been reported on wild plants and was expected to be enhanced due to perennial host plants and generalist vectors (Tugume *et al.* 2016). Bacterial interspecific infections of eight *Pseudomonas spp.* have been reported to cause more severe infections in tomato (Lamichhane and Venturi 2015). Comprehensive understanding of how synergism between different pathogens leads to higher disease severity is limited, however may be more frequently occurring than realized (Lamichhane and Venturi 2015). Variation of our data may also be attributed to variable temperatures that the batches were conducted in through the months of August through November. The development and progression of bacterial leaf spot has been shown to thrive in environmental conditions with high humidity and high temperatures and the importance of climate change has also been indicated on mixed infection severity (Abrahamian *et al.,* 2021; Guerret *et al*. 2016).

In conclusion, our study suggests a trend in which the number of genotypes present in a niche or co-infection leads to higher severity and AUDPC values when more diversity is introduced (Figure 3-1, 3-4, and 3-5). Further evaluation of individual strain fitness within a mixed infection, effector production, and comparison of severity indices in the field may provide further context behind these co-infections and the mediators behind the interactions. The understanding and establishment of these trends can be utilized as a vital tool in predicting disease severity of a field based on the intraspecific variation observed and accounted for.

References

Abrahamian, P., Timilsina, S., Minsavage, G. V., Kc, S., Goss, E. M., Jones, J. B., & Vallad, G. E. (2018). The type III effector AvrBsT enhances Xanthomonas perforans fitness in field-grown tomato. Phytopathology, 108(12), 1355-1362.

Abrahamian, Peter, et al. "Epidemiology, diversity, and management of bacterial spot of tomato caused by Xanthomonas perforans." Applied Microbiology and Biotechnology 105.16 (2021): 6143-6158.

Alvarez-Martinez, C. E., Sgro, G. G., Araujo, G. G., Paiva, M. R., Matsuyama, B. Y., Guzzo, C. R., ... & Farah, C. S. (2021). Secrete or perish: The role of secretion systems in Xanthomonas biology. Computational and Structural Biotechnology Journal, 19, 279-302.

Barak, J. D., Vancheva, T., Lefeuvre, P., Jones, J. B., Timilsina, S., Minsavage, G. V., ... & Koebnik, R. (2016). Whole-genome sequences of Xanthomonas euvesicatoria strains clarify taxonomy and reveal a stepwise erosion of type 3 effectors. *Frontiers in Plant Science*, *7*, 1805.

Barrett, L. G., Zala, M., Mikaberidze, A., Alassimone, J., Ahmad, M., McDonald, B. A., & Sánchez-Vallet, A. (2021). Mixed infections alter transmission potential in a fungal plant pathogen. Environmental microbiology, 23(4), 2315-2330.

Bates, D., Sarkar, D., Bates, M. D., & Matrix, L. (2007). The lme4 package. R package version, 2(1), 74.

Bhattarai, K., Louws, F. J., Williamson, J. D., & Panthee, D. R. (2016). Differential response of tomato genotypes to Xanthomonas-specific pathogen-associated molecular patterns and correlation with bacterial spot (Xanthomonas perforans) resistance. *Horticulture*

research, *3*.

Bulgarelli, D., Schlaeppi, K., Spaepen, S., Van Themaat, E. V. L., & Schulze-Lefert, P. (2013). Structure and functions of the bacterial microbiota of plants. Annual review of plant biology, 64, 807-838.

Chiang, K. S., & Bock, C. H. (2021). Understanding the ramifications of quantitative ordinal scales on accuracy of estimates of disease severity and data analysis in plant pathology. Tropical Plant Pathology, 1-16.

Colhoun, J. (1973). Effects of environmental factors on plant disease. *Annual review of phytopathology*, *11*(1), 343-364.

Guerret, M. G., Barbetti, M. J., You, M. P., & Jones, R. A. (2016). Effects of temperature on disease severity in plants of subterranean clover infected singly or in mixed infection with Bean yellow mosaic virus and Kabatiella caulivora. Journal of Phytopathology, 164(9), 608-619.

Hollis, J. P. (1984). The Horsfall-Barratt Grading System. Plant pathology, 33(2), 145-146.

Jacques, M. A., Arlat, M., Boulanger, A., Boureau, T., Carrère, S., Cesbron, S., ... & Vernière, C. (2016). Using ecology, physiology, and genomics to understand host specificity in Xanthomonas. Annual review of phytopathology, 54, 163-187.

Kinnula, H., Mappes, J., & Sundberg, L. R. (2017). Coinfection outcome in an opportunistic pathogen depends on the inter-strain interactions. BMC evolutionary biology, 17(1), 1-10.

Klein-Gordon, J. M., Xing, Y., Garrett, K. A., Abrahamian, P., Paret, M. L., Minsavage, G. V., ... & Vallad, G. E. (2021). Assessing changes and associations in the Xanthomonas perforans population across Florida commercial tomato fields via a statewide

survey. Phytopathology®, 111(6), 1029-1041.

Klein-Gordon, J. M., Timilsina, S., Xing, Y., Abrahamian, P., Garrett, K. A., Jones, J. B., ... & Goss, E. M. (2022). Whole genome sequences reveal the Xanthomonas perforans population is shaped by the tomato production system. The ISME journal, 16(2), 591-601.

Kranz, J. (1988). Measuring plant disease. In *Experimental techniques in plant disease epidemiology* (pp. 35-50). Springer, Berlin, Heidelberg.

Kuznetsova, A., Brockhoff, P. B., & Christensen, R. H. B. (2015). Package 'lmertest'. R package version, 2(0), 734.

Lamichhane, J. R., & Venturi, V. (2015). Synergisms between microbial pathogens in plant disease complexes: a growing trend. Frontiers in plant science, 6, 385.

Lewis Ivey, M. L., Strayer, A., Sidhu, J. K., & Minsavage, G. V. (2016). Bacterial leaf spot of tomato (Solanum lycopersicum) in Louisiana is caused by Xanthomonas perforans, tomato race 4. Plant Disease, 100(6), 1233-1233.

Lin, R. H., Peng, C. W., Lin, Y. C., Peng, H. L., & Huang, H. C. (2011). The XopE2 effector protein of Xanthomonas campestris pv. vesicatoria is involved in virulence and in the suppression of the hypersensitive response. *Botanical studies*, *52*(1).

Mbega, E. R., Mabagala, R. B., Adriko, J., Lund, O. S., Wulff, E. G., & Mortensen, C. N. (2012). Five species of xanthomonads associated with bacterial leaf spot symptoms in tomato from Tanzania. Plant Disease, 96(5), 760-760.

Mideo, N. (2009). Parasite adaptations to within-host competition. Trends in parasitology, 25(6), 261-268.

Moreno, A. B., & López-Moya, J. J. (2020). When viruses play team sports: mixed infections in

plants. Phytopathology, 110(1), 29-48.

Newberry, E. A., Bhandari, R., Minsavage, G. V., Timilsina, S., Jibrin, M. O., Kemble, J., ... & Potnis, N. (2019). Independent evolution with the gene flux originating from multiple Xanthomonas species explains genomic heterogeneity in Xanthomonas perforans. *Applied and environmental microbiology*, *85*(20), e00885-19.

Newberry, E., Bhandari, R., Kemble, J., Sikora, E., & Potnis, N. (2020). Genome-resolved metagenomics to study co-occurrence patterns and intraspecific heterogeneity among plant pathogen metapopulations. *Environmental Microbiology*, *22*(7), 2693-2708.

Potnis, N., Timilsina, S., Strayer, A., Shantharaj, D., Barak, J. D., Paret, M. L., ... & Jones, J. B. (2015). Bacterial spot of tomato and pepper: diverse Xanthomonas species with a wide variety of virulence factors posing a worldwide challenge. Molecular plant pathology, 16(9), 907-920.

Rochow, W. F. (1972). The role of mixed infections in the transmission of plant viruses by aphids. Annual Review of Phytopathology, 10(1), 101-124.

Rowe, H. C., & Kliebenstein, D. J. (2010). All mold is not alike: the importance of intraspecific diversity in necrotrophic plant pathogens. PLoS pathogens, 6(3), e1000759.

Simko, I., & Piepho, H. P. (2012). The area under the disease progress stairs: calculation, advantage, and application. Phytopathology, 102(4), 381-389.

Strange, R. N. (2003). Introduction to plant pathology. John Wiley & Sons.

Stubbendieck, R. M., & Straight, P. D. (2016). Multifaceted Interfaces of Bacterial Competition. Journal of Bacteriology, 198(16), 2145–2155. https://doi.org/10.1128/JB.00275-16.

Susi, H., Barrès, B., Vale, P. F., & Laine, A. L. (2015). Co-infection alters population dynamics of infectious disease. Nature communications, 6(1), 1-8.

Tugume, A. K., Mukasa, S. B., & Valkonen, J. P. (2016). Mixed infections of four viruses, the incidence and phylogenetic relationships of Sweet potato chlorotic fleck virus (Betaflexiviridae) isolates in wild species and sweetpotatoes in Uganda and evidence of distinct isolates in East Africa. PLoS One, 11(12), e0167769.

White, F. F., Potnis, N., Jones, J. B., & Koebnik, R. (2009). The type III effectors of Xanthomonas. Molecular plant pathology, 10(6), 749-766.

Wickham, H., Francois, R., Henry, L., & Müller, K. (2014, June). Dplyr. In useR! Conference.

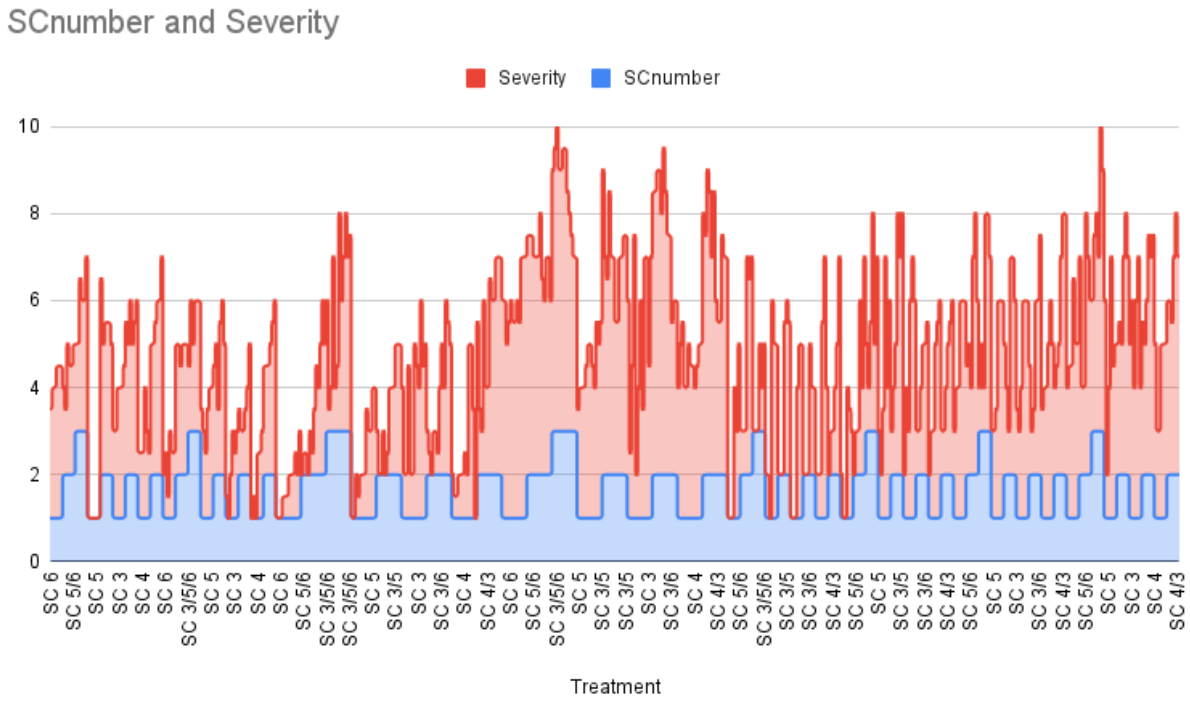


Figure 3-1: **Number of genotypes within an infection reveals trend of higher disease severity.**

Stacked-step area chart representing levels of disease severity based on treatments among the number of sequence clusters utilized per infection. Blue step area identifies the number of sequence clusters/ genotypes present in the infection. Red step area identifies the correlated disease severity index to the treatment. Repetitive treatments are indicative of different batches.

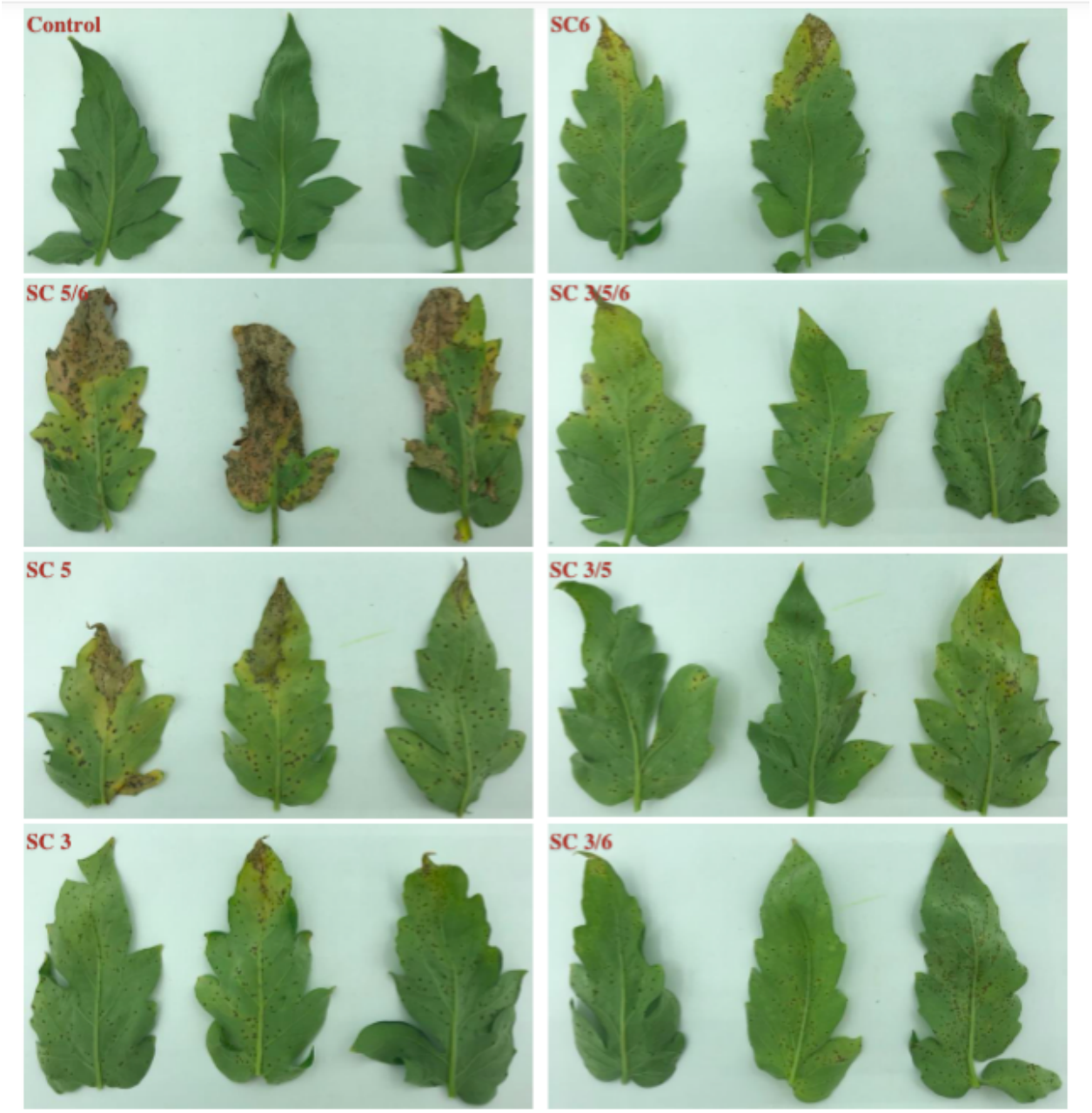


Figure 3-2: Symptomatic tissue of mixed genotype infections.

Effect of single and multiple coinfection treatments by *Xanthomonas perforans* sequence clusters/genotypes on representative leaves from symptomatic leaf tissue of batch one in August 2021. Disease severity observed at fourteen days post inoculation with ~1 X 10^6 cell suspensions of infections with SC 6, SC 5/6, SC 3/5/6, SC 5, SC 3/5, SC 3, and SC 3/6.

Figure 3-3: AUDPC of mixed genotype infections for individual batches.

Area under disease progress curve (AUDPC) plotted for each treatment across seven experimental batches. AUDPC raw values and means are estimated by a linear mixed model with 95% confidence interval per strain(s).

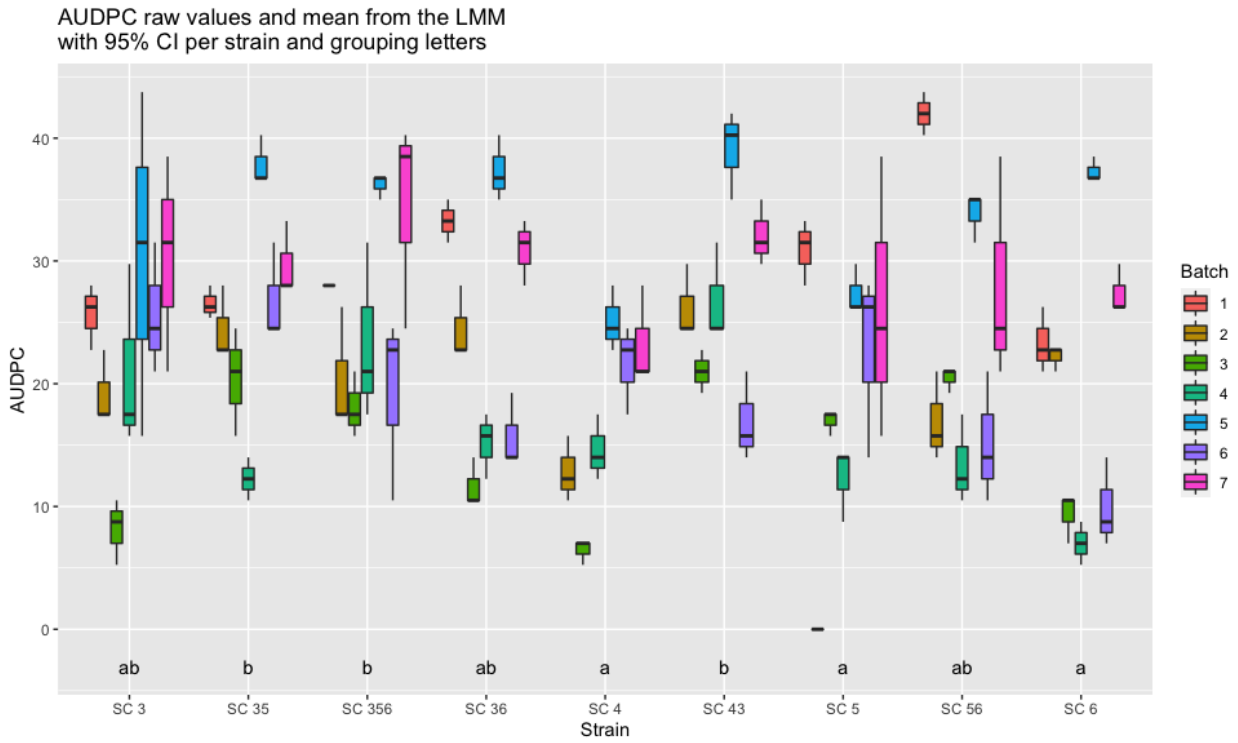


Figure 3-4: AUDPC of mixed genotype infections with batch effect.

Seven experimental batches are displayed for each of the genotype infections including, SC 3, SC 3/5, SC 3/5/6, SC 3/6, SC 4, SC 4/3, SC 5, SC 5/6, SC 6. Area under the disease progress curve (AUDPC) calculated raw values are indicated by the box whisker plot. AUDPC raw values and means from linear mixed model with 95% confidence interval (CI) per strain(s). Letters (ab, b, a) indicate statistical significance based on Tukey. Individual batches for each treatment are indicated by the colored legend. Thick horizontal lines represent means and colored boxes represent 95% CI.

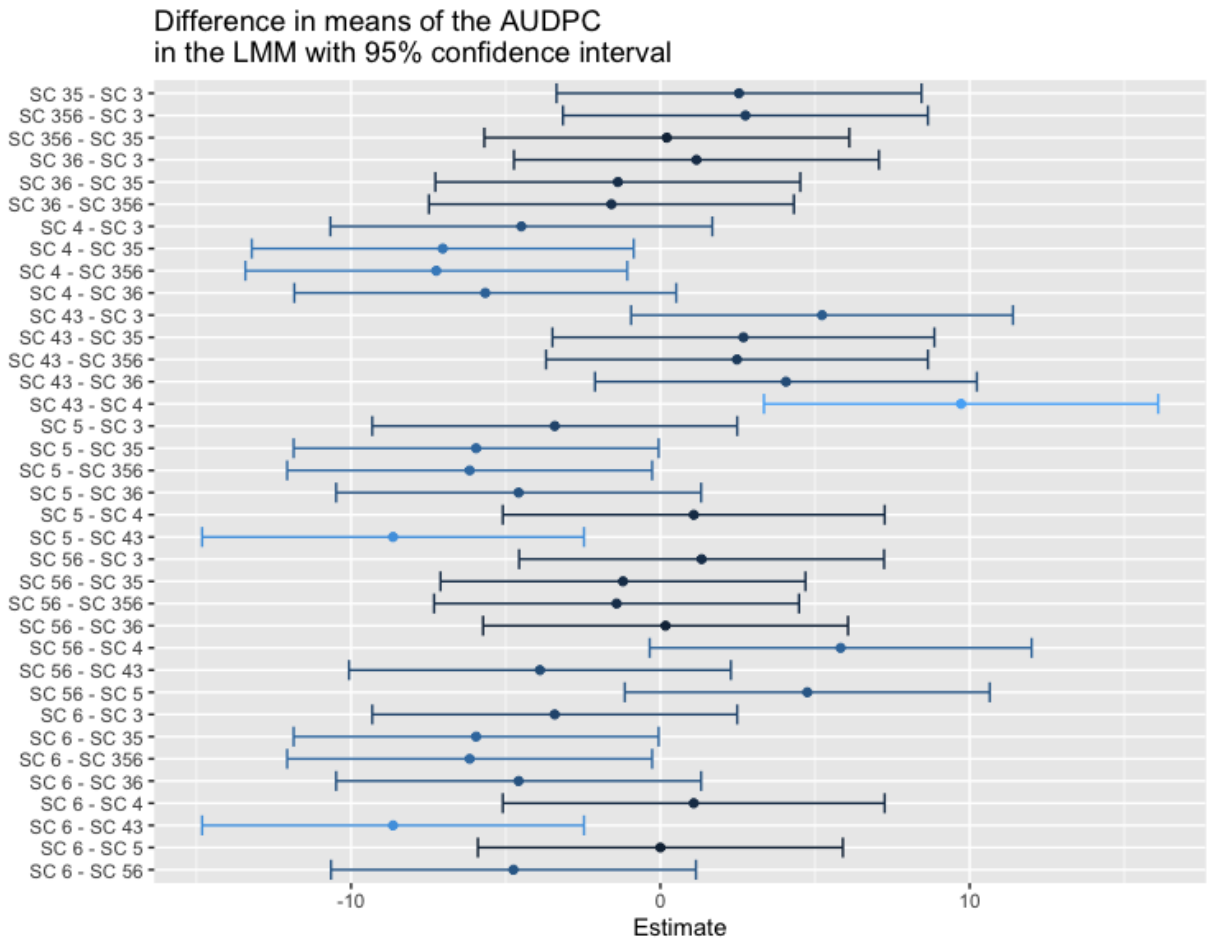


Figure 3-5: Difference in means of the AUDPC of mixed genotype infections.

Mean values of the AUDPC in the linear mixed model (LMM) with a 95% confidence interval were contrasted between treatments. Difference in the means of the AUDPC with an estimate 0 have no difference in the mean of AUDPC among pair of strains. Negative and positive values represent statistically significant treatments based on Tukey.

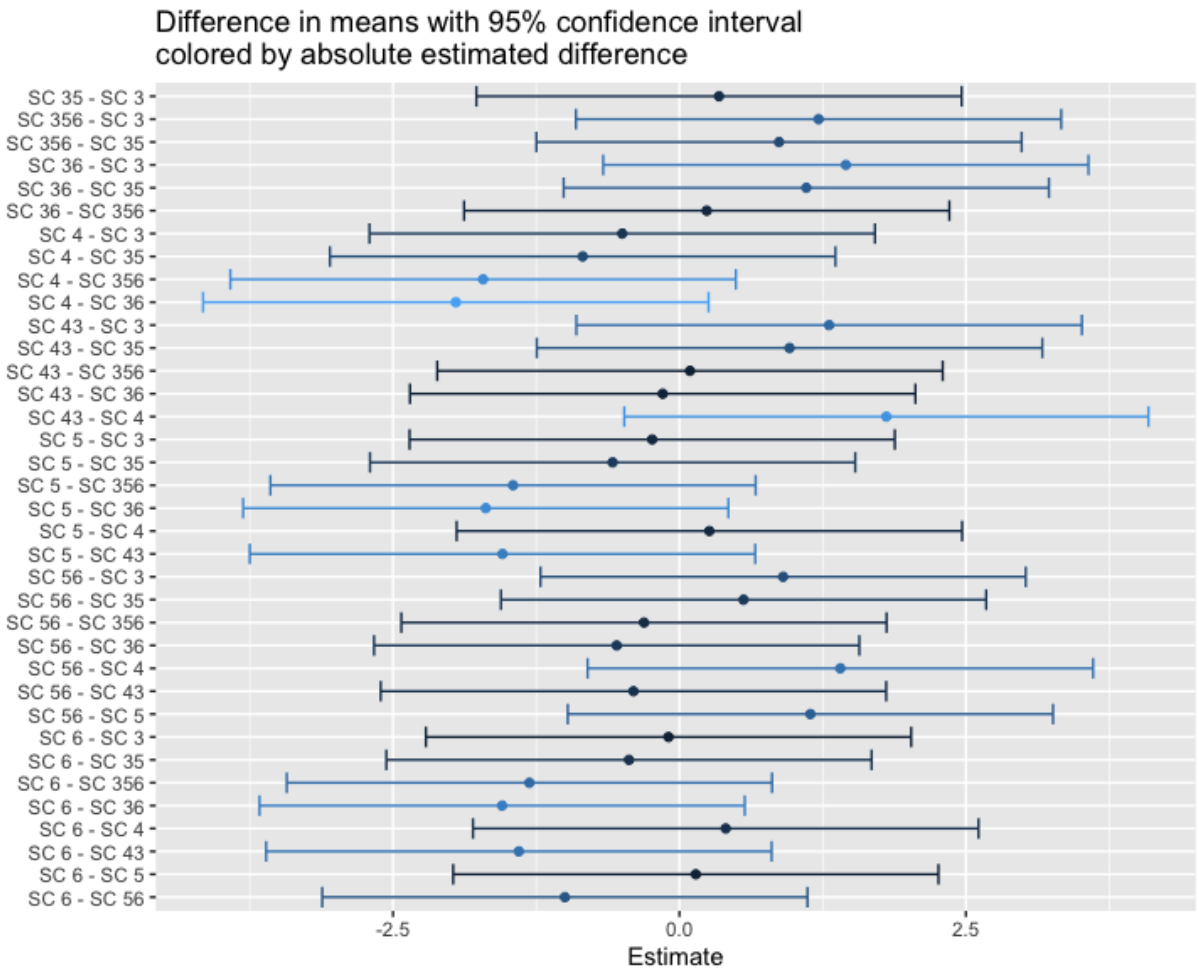


Figure 3-6: Difference in means of disease development of mixed genotype infections.

Mean values of disease development with a 95% confidence interval were contrasted between treatments. Difference in means of disease development between treatments with an estimate 0 have no difference in means of disease development among pair of strains. Negative and positive values represent statistically significant treatments based on Tukey.

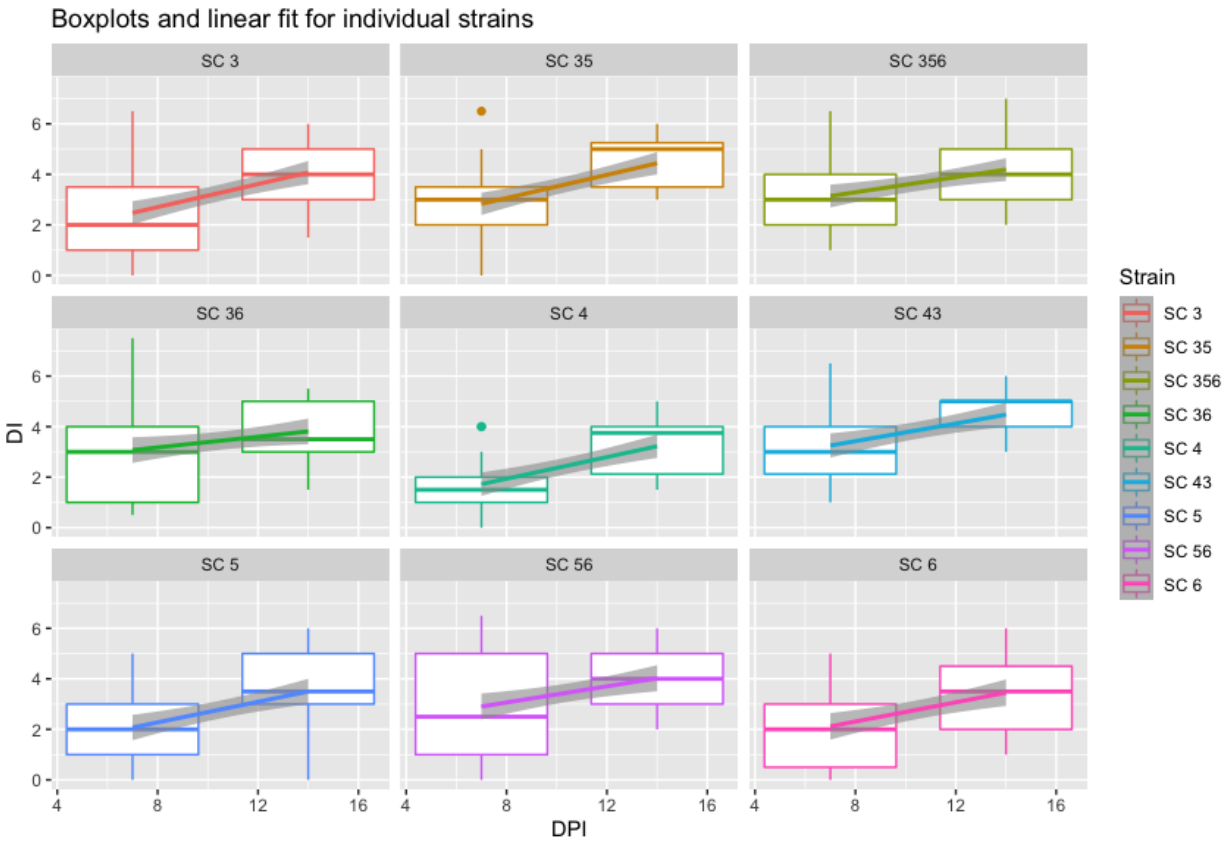


Figure 3-7: Linear fit model for AUDPC between mixed genotype infections.

Linear fit model for individual treatments at day 7 and day 14. Plants were dip-inoculated with ~1 X 10^6 of cell suspensions for each treatment and evaluated for disease severity. Slopes of AUDPC vary between treatments indicating varying disease development.

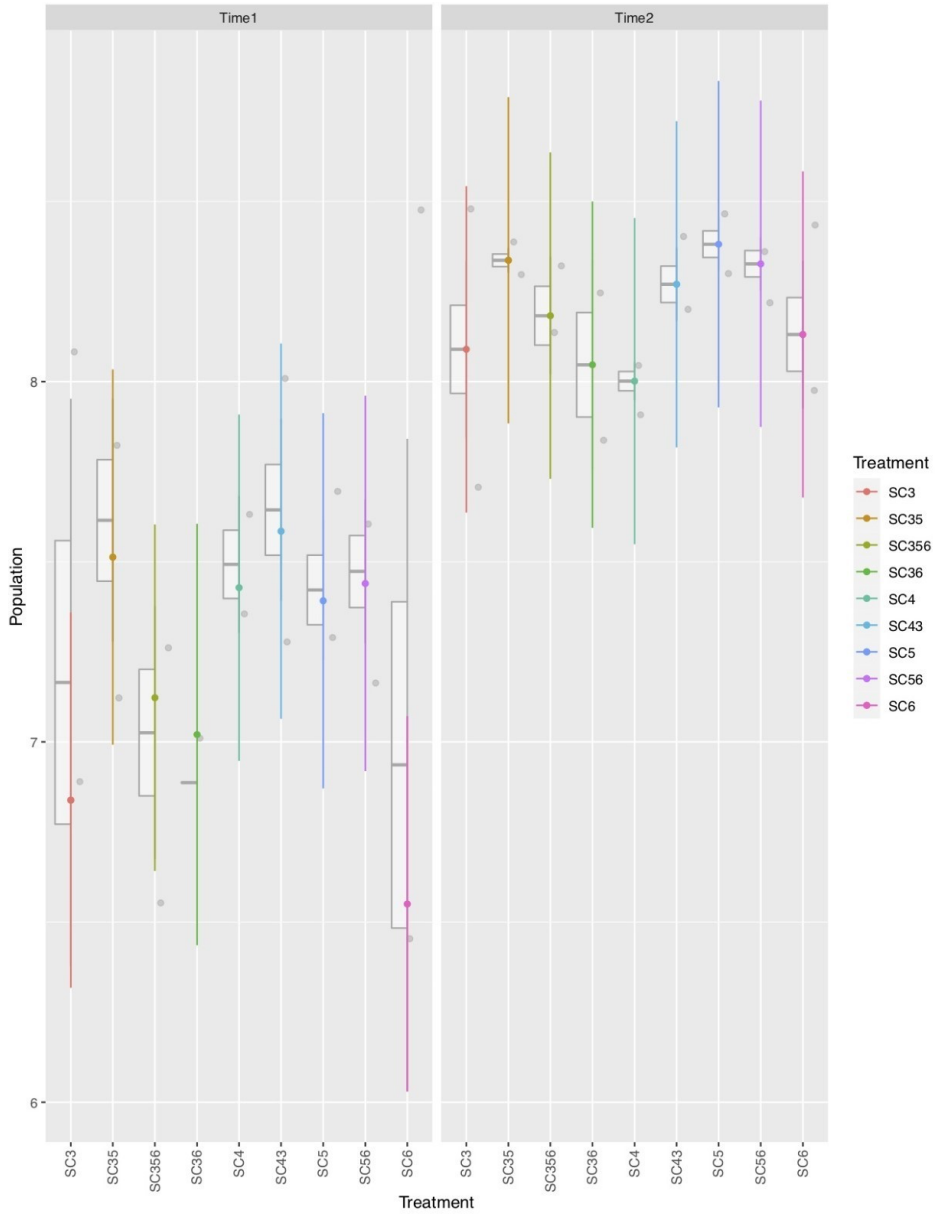


Figure 3-8: Effect of co-infection treatments on Xp genotype population.

Four- to five-week-old tomato (cv. FL47) plants were inoculated with ~1x 10^6 cfu/ml of SC3, SC3/5, SC3/5/6, SC3/6, SC4, SC4/3, SC5, SC5/6, and SC6. Growth of Xp genotype population was evaluated from plants inoculated with the different treatments on day 7 (Time 1) and day 14 (Time 2). Mixed linear model was applied for the statistical analysis of the $\log_{10}$ cfu/cm^2 of Xp values. Tukey's test of least significant difference ($P < 0.05$) revealed no significance.

	SC 3 Xp2010	SC 4 AL57	SC 5 AL37	SC 6 AL65
AvrXv3	-	+	+	IS
XopAQ	-	+	+	-
PthXp1	-	+	+	-
AvrBsT	-	+	+	-
XopE3	-	IS	IS	-
XopE2	-	+	-	-
AvrHah1	-	-	-	+
XopAO	-	-	-	+

Table 3-1: Effector representation among representative strains.

Table representing the presence (+) or absence (-), or insertion sequence (IS) of eight type III secretion variable effectors in the representative strains of this study. SC 3 has an inactivated AvrXv3 gene and a lack of representation for the following seven effector genes. SC 4 has a presence of effector genes, AvrXv3, XopAQ, PthXp1, AvrBsT, and XopE2 with an insertion sequence present in the midst of XopE3. SC5 has a presence of effector genes, AvrXv3, XopAQ, PthXp1, AvrBsT, with an insertion sequence present in the midst of XopE3. SC 5 has a presence of effector genes, AvrHah1 and XopAO with an insertion sequence present in the midst of AvrXv3.

4. REFERENCES

Abrahamian, P., Timilsina, S., Minsavage, G. V., Kc, S., Goss, E. M., Jones, J. B., & Vallad, G. E. (2018). The type III effector AvrBsT enhances Xanthomonas perforans fitness in field-grown tomato. Phytopathology, 108(12), 1355-1362.

Abrahamian, P., Klein-Gordon, J. M., Jones, J. B., & Vallad, G. E. (2021). Epidemiology, diversity, and management of bacterial spot of tomato caused by Xanthomonas perforans. Applied Microbiology and Biotechnology, 105(16), 6143-6158.

Allsopp, L. P., Bernal, P., Nolan, L. M., & Filloux, A. (2020). Causalities of war: the connection between type VI secretion system and microbiota. Cellular microbiology, 22(3), e13153.

Alvarez-Martinez, C. E., Sgro, G. G., Araujo, G. G., Paiva, M. R., Matsuyama, B. Y., Guzzo, C. R., ... & Farah, C. S. (2021). Secrete or perish: The role of secretion systems in Xanthomonas biology. Computational and Structural Biotechnology Journal, 19, 279-302.

Barak, J. D., Vancheva, T., Lefeuvre, P., Jones, J. B., Timilsina, S., Minsavage, G. V., ... & Koebnik, R. (2016). Whole-genome sequences of Xanthomonas euvesicatoria strains clarify taxonomy and reveal a stepwise erosion of type 3 effectors. *Frontiers in Plant Science*, *7*, 1805.

Barrett, L. G., Zala, M., Mikaberidze, A., Alassimone, J., Ahmad, M., McDonald, B. A., & Sánchez-Vallet, A. (2021). Mixed infections alter transmission potential in a fungal plant pathogen. Environmental microbiology, 23(4), 2315-2330.

Bashir, I., War, A. F., Rafiq, I., Reshi, Z. A., Rashid, I., & Shouche, Y. S. (2021). Phyllosphere microbiome: Diversity and functions. Microbiological Research, 126888.

Bates, D., Sarkar, D., Bates, M. D., & Matrix, L. (2007). The lme4 package. R package version, 2(1), 74.

Bernal, P., Llamas, M. A., & Filloux, A. (2018). Type VI secretion systems in plant associated bacteria. Environmental microbiology, 20(1), 1-15.

Bernal, P., Allsopp, L. P., Filloux, A., & Llamas, M. A. (2017). The Pseudomonas putida T6SS is a plant warden against phytopathogens. The ISME journal, 11(4), 972-987.

Bhattarai, K., Louws, F. J., Williamson, J. D., & Panthee, D. R. (2016). Differential response of tomato genotypes to Xanthomonas-specific pathogen-associated molecular patterns and correlation with bacterial spot (Xanthomonas perforans) resistance. *Horticulture research*, *3*.

Boch, J., & Bonas, U. (2010). Xanthomonas AvrBs3 family-type III effectors: discovery and function. Annual review of phytopathology, 48, 419-436.

Bulgarelli, D., Schlaeppi, K., Spaepen, S., Van Themaat, E. V. L., & Schulze-Lefert, P. (2013). Structure and functions of the bacterial microbiota of plants. Annual review of plant biology, 64, 807-838.

Chiang, K. S., & Bock, C. H. (2021). Understanding the ramifications of quantitative ordinal scales on accuracy of estimates of disease severity and data analysis in plant pathology. Tropical Plant Pathology, 1-16.

Choi, Y., Kim, N., Mannaa, M., Kim, H., Park, J., Jung, H., ... & Seo, Y. S. (2020). Characterization of type VI secretion system in Xanthomonas oryzae pv. oryzae and its role in virulence to rice. The plant pathology journal, 36(3), 289.

Colhoun, J. (1973). Effects of environmental factors on plant disease. *Annual review of phytopathology*, *11*(1), 343-364.

Dong, C. J., Wang, L. L., Li, Q., & Shang, Q. M. (2019). Bacterial communities in the rhizosphere, phyllosphere and endosphere of tomato plants. PLoS One, 14(11), e0223847.

Dong, C., Wang, L., Li, Q., & Shang, Q. (2021). Epiphytic and endophytic fungal communities of tomato plants. Horticultural Plant Journal, 7(1), 38-48.

Enya, J., Shinohara, H., Yoshida, S., Tsukiboshi, T., Negishi, H., Suyama, K., & Tsushima, S. (2007). Culturable leaf-associated bacteria on tomato plants and their potential as biological control agents. Microbial ecology, 53(4), 524-536.

Gallegos-Monterrosa, R., & Coulthurst, S. J. (2021). The ecological impact of a bacterial weapon: microbial interactions and the Type VI secretion system. FEMS Microbiology Reviews, 45(6), fuab033.

Gong, T., & Xin, X. F. (2021). Phyllosphere microbiota: Community dynamics and its interaction with plant hosts. Journal of Integrative Plant Biology, 63(2), 297-304.

Guerret, M. G., Barbetti, M. J., You, M. P., & Jones, R. A. (2016). Effects of temperature on disease severity in plants of subterranean clover infected singly or in mixed infection with Bean yellow mosaic virus and Kabatiella caulivora. Journal of Phytopathology, 164(9), 608-619.

Gupta, S., Ray, S., Khan, A., China, A., Das, D., & Mallick, A. I. (2021). The cost of bacterial predation via type VI secretion system leads to predator extinction under environmental stress. Iscience, 24(12), 103507.

Hachani, A., Lossi, N. S., & Filloux, A. (2013). A visual assay to monitor T6SS-mediated bacterial competition. Journal of visualized experiments: JoVE, (73).

Hollis, J. P. (1984). The Horsfall-Barratt Grading System. Plant pathology, 33(2), 145-146.

Jacques, M. A., Arlat, M., Boulanger, A., Boureau, T., Carrère, S., Cesbron, S., ... & Vernière, C. (2016). Using ecology, physiology, and genomics to understand host specificity in Xanthomonas. Annual review of phytopathology, 54, 163-187.

Jani, A. J., & Cotter, P. A. (2010). Type VI secretion: not just for pathogenesis anymore. *Cell host & microbe*, *8*(1), 2-6.

Kinnula, H., Mappes, J., & Sundberg, L. R. (2017). Coinfection outcome in an opportunistic pathogen depends on the inter-strain interactions. BMC evolutionary biology, 17(1), 1-10.

Klein-Gordon, J. M., Xing, Y., Garrett, K. A., Abrahamian, P., Paret, M. L., Minsavage, G. V., ... & Vallad, G. E. (2021). Assessing changes and associations in the Xanthomonas perforans population across Florida commercial tomato fields via a statewide survey. Phytopathology®, 111(6), 1029-1041.

Klein-Gordon, J. M., Timilsina, S., Xing, Y., Abrahamian, P., Garrett, K. A., Jones, J. B., ... & Goss, E. M. (2022). Whole genome sequences reveal the Xanthomonas perforans population is shaped by the tomato production system. The ISME journal, 16(2), 591-601.

Kranz, J. (1988). Measuring plant disease. In *Experimental techniques in plant disease epidemiology* (pp. 35-50). Springer, Berlin, Heidelberg.

Kuznetsova, A., Brockhoff, P. B., & Christensen, R. H. B. (2015). Package 'lmertest'. R package version, 2(0), 734.

Lamichhane, J. R., & Venturi, V. (2015). Synergisms between microbial pathogens in plant disease complexes: a growing trend. Frontiers in plant science, 6, 385.

Lemanceau, P., Barret, M., Mazurier, S., Mondy, S., Pivato, B., Fort, T., & Vacher, C. (2017). Plant communication with associated microbiota in the spermosphere, rhizosphere and phyllosphere. Advances in botanical research, 82, 101-133.

Lewis Ivey, M. L., Strayer, A., Sidhu, J. K., & Minsavage, G. V. (2016). Bacterial leaf spot of tomato (Solanum lycopersicum) in Louisiana is caused by Xanthomonas perforans, tomato race 4. Plant Disease, 100(6), 1233-1233.

Lin, R. H., Peng, C. W., Lin, Y. C., Peng, H. L., & Huang, H. C. (2011). The XopE2 effector protein of Xanthomonas campestris pv. vesicatoria is involved in virulence and in the suppression of the hypersensitive response. *Botanical studies*, *52*(1).

Liu, H., Brettell, L. E., & Singh, B. (2020). Linking the phyllosphere microbiome to plant health. Trends in Plant Science, 25(9), 841-844. Trends in Plant Science, 25(9), 841-844.

Liyanapathiranage, P., Jones, J. B., & Potnis, N. (2021). A mutation of a single core gene, tssM, of Type VI secretion system of Xanthomonas perforans influences virulence, epiphytic survival and transmission during pathogenesis on tomato. Phytopathology, (ja).

Mbega, E. R., Mabagala, R. B., Adriko, J., Lund, O. S., Wulff, E. G., & Mortensen, C. N. (2012). Five species of xanthomonads associated with bacterial leaf spot symptoms in tomato from Tanzania. Plant Disease, 96(5), 760-760.

Mideo, N. (2009). Parasite adaptations to within-host competition. Trends in parasitology, 25(6), 261-268.

Montenegro Benavides, N. A., Alvarez B, A., Arrieta-Ortiz, M. L., Rodriguez-R, L. M., Botero, D., Tabima, J. F., ... & Bernal, A. (2021). The type VI secretion system of Xanthomonas phaseoli pv. manihotis is involved in virulence and in vitro motility. BMC microbiology, 21(1), 1-12.

Moreno, A. B., & López-Moya, J. J. (2020). When viruses play team sports: mixed infections in plants. Phytopathology, 110(1), 29-48.

Müller, T., & Ruppel, S. (2014). Progress in cultivation-independent phyllosphere microbiology. FEMS microbiology ecology, 87(1), 2-17.

Newberry, E. A., Bhandari, R., Minsavage, G. V., Timilsina, S., Jibrin, M. O., Kemble, J., ... & Potnis, N. (2019). Independent evolution with the gene flux originating from multiple Xanthomonas species explains genomic heterogeneity in Xanthomonas perforans. Applied and environmental microbiology, 85(20), e00885-19.

Newberry, E., Bhandari, R., Kemble, J., Sikora, E., & Potnis, N. (2020). Genome-resolved metagenomics to study co-occurrence patterns and intraspecific heterogeneity among plant pathogen metapopulations. *Environmental microbiology*, *22*(7), 2693-2708.

Ottesen, A. R., Gorham, S., Reed, E., Newell, M. J., Ramachandran, P., Canida, T., ... & White, J. R. (2016). Using a control to better understand phyllosphere microbiota. PLoS one,11(9), e0163482.

Ottesen, A. R., Gonzalez, A., Bell, R., Arce, C., Rideout, S., Allard, M., ... & Pettengill, J. B. (2013). Co-enriching microflora associated with culture based methods to detect Salmonella from tomato phyllosphere. PloS one, 8(9), e73079.

Potnis, N., Timilsina, S., Strayer, A., Shantharaj, D., Barak, J. D., Paret, M. L., ... & Jones, J. B. (2015). Bacterial spot of tomato and pepper: diverse Xanthomonas species with a wide variety of virulence factors posing a worldwide challenge. Molecular plant pathology, 16(9), 907-920.

Rastogi, G., Coaker, G. L., & Leveau, J. H. (2013). New insights into the structure and function of phyllosphere microbiota through high-throughput molecular approaches. FEMS microbiology letters, 348(1), 1-10.

Rochow, W. F. (1972). The role of mixed infections in the transmission of plant viruses by aphids. Annual Review of Phytopathology, 10(1), 101-124.

Rowe, H. C., & Kliebenstein, D. J. (2010). All mold is not alike: the importance of intraspecific diversity in necrotrophic plant pathogens. PLoS pathogens, 6(3), e1000759.

Russell, A. B., Peterson, S. B., & Mougous, J. D. (2014). Type VI secretion system effectors: poisons with a purpose. Nature reviews microbiology, 12(2), 137-148.

Schloss, P. D. (2020). Reintroducing mothur: 10 years later. Applied and Environmental Microbiology, 86(2), e02343-19.

Senthilkumar, M., & Krishnamoorthy, R. (2017). Isolation and characterization of tomato leaf phyllosphere Methylobacterium and their effect on plant growth. Int J Curr Microbiol App Sci, 6(11), 2121-2136

Sharma, A., Timilsina, S., Abrahamian, P., Minsavage, G. V., Colee, J., Ojiambo, P. S., ... & Jones, J. B. (2021). Need for speed: bacterial effector XopJ2 is associated with increased dispersal velocity of Xanthomonas perforans. Environmental Microbiology.

Shyntum, D. Y., Nkomo, N. P., Shingange, N. L., Gricia, A. R., Bellieny-Rabelo, D., & Moleleki, L. N. (2019). The impact of type VI secretion system, bacteriocins and antibiotics on bacterial competition of Pectobacterium carotovorum subsp. brasiliense and the regulation of carbapenem biosynthesis by iron and the ferric-uptake regulator. Frontiers in microbiology, 2379.

Silverman, J. M., Brunet, Y. R., Cascales, E., & Mougous, J. D. (2012). Structure and regulation

of the type VI secretion system. Annual review of microbiology, 66, 453-472.

Simko, I., & Piepho, H. P. (2012). The area under the disease progress stairs: calculation, advantage, and application. Phytopathology, 102(4), 381-389.

Sivakumar, N., Sathishkumar, R., Selvakumar, G., Shyamkumar, R., & Arjunekumar, K. (2020). Phyllospheric microbiomes: diversity, ecological significance, and biotechnological applications. Plant Microbiomes for Sustainable Agriculture, 25, 113.

Smith, W. P., Brodmann, M., Unterweger, D., Davit, Y., Comstock, L. E., Basler, M., & Foster, K. R. (2020). The evolution of tit-for-tat in bacteria via the type VI secretion system. *Nature communications*, *11*(1), 1-11.

Soria-Bustos, J., Ares, M. A., Gómez-Aldapa, C. A., González-y-Merchand, J. A., Girón, J. A., & De la Cruz, M. A. (2020). Two type VI secretion systems of Enterobacter cloacae are required for bacterial competition, cell adherence, and intestinal colonization. *Frontiers in microbiology*, 2137.

Strange, R. N. (2003). Introduction to plant pathology. John Wiley & Sons.

Stromberg, K. D., Kinkel, L. L., & Leonard, K. J. (2004). Quantifying the effect of bacterial antagonists on the relationship between phyllosphere population sizes of Xanthomonas translucens pv. translucens and subsequent bacterial leaf streak severity on wheat seedlings. Biological Control, 29(1), 58-65.

Stubbendieck, R. M., & Straight, P. D. (2016). Multifaceted Interfaces of Bacterial Competition. Journal of Bacteriology, 198(16), 2145–2155. https://doi.org/10.1128/JB.00275-16.

Sundin, G. W. (2006). Host-pathogen interactions of relevance to the phyllosphere. Microbial ecology of aerial plant surfaces, 201.

Susi, H., Barrès, B., Vale, P. F., & Laine, A. L. (2015). Co-infection alters population

dynamics of infectious disease. Nature communications, 6(1), 1-8.

Thapa, S., & Prasanna, R. (2018). Prospecting the characteristics and significance of the phyllosphere microbiome. Annals of microbiology, 68(5), 229-245.

Timilsina, S., Abrahamian, P., Potnis, N., Minsavage, G. V., White, F. F., Staskawicz, B. J., ... & Goss, E. M. (2016). Analysis of sequenced genomes of Xanthomonas perforans identifies candidate targets for resistance breeding in tomato. Phytopathology, 106(10), 1097-1104.

Tugume, A. K., Mukasa, S. B., & Valkonen, J. P. (2016). Mixed infections of four viruses, the incidence and phylogenetic relationships of Sweet potato chlorotic fleck virus (Betaflexiviridae) isolates in wild species and sweetpotatoes in Uganda and evidence of distinct isolates in East Africa. PLoS One, 11(12), e0167769.

White, F. F., Potnis, N., Jones, J. B., & Koebnik, R. (2009). The type III effectors of Xanthomonas. Molecular plant pathology, 10(6), 749-766.

Wickham, H., Francois, R., Henry, L., & Müller, K. (2014, June). Dplyr. In useR! Conference.

Zhang, B., Bai, Z., Hoefel, D., Wang, X., Zhang, L., & Li, Z. (2010). Microbial diversity within the phyllosphere of different vegetable species. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, 2, 1067-1077.

Zhang, Y., Callaway, E. M., Jones, J. B., & Wilson, M. (2009). Visualisation of hrp gene expression in Xanthomonas euvesicatoria in the tomato phyllosphere. European journal of plant pathology, 124(3), 379-390.