Differential Strain Dynamics Under Host and Environmental Stressors: A Pathway to Better Understanding Pathogen Presence and Fitness of *Xanthomonas perforans*

By

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Abstract

The bacterium *Xanthomonas perforans* is a worldwide pathogen of tomato and pepper plants causing bacterial leaf spot (BLS) resulting in necrotic spotting with which advanced disease can lead to complete defoliation of plants and subsequent yield loss for farmers. BLS is a seedborne disease that is considered endemic to the Southeastern United States, and while management strategies focus on the creation and use of resistant (R) cultivars in pepper, no R tomato cultivars are available in tomato due to the range of genetic diversity and subsequent difficulty of breeding for single-gene resistance. To understand this pathosystem better with hopes of aiding in the development of better disease management practices, we firstly profiled *X. perforans* pathogen diversity and disease dynamics across the Southeastern United States. We found that the pathogen was intraspecifically heterogenous within fields and across space and time, that the pathogen population had an effect on disease outcomes, and that climatic parameters were associated with disease and pathogen dynamics. These findings were then narrowed down in a greenhouse experiment to study host:pathogen and pathogen:pathogen interactions where we found that pathogen population assemblage was determined irrespective of environment. Lastly, we conducted research in light of global changes occurring by studying *X. perforans* under an extreme environmental variable to understand more about pathogen evolution across a single growing season and found that when in a combination of stress with host resistance and an environmental extreme, pathogen and disease dynamics shifted and an adaptive response was observed at a genomic level. With a look into pathogen dynamics in the field and implications of how those dynamics might change with environmental extremes, this work advances plant pathological research of the diverse and adaptive *Xanthomonas perforans*.

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Table of Contents

List of Tables

List of Figures

1. CHAPTER ONE

Introduction and Literature Review

Introduction

While the field of plant pathology often goes unnoticed by the general public, a large basis of plant pathological work focuses on one important fact: national food security essentially underpins all functions of the society, yet is threatened by a continuous and evolving pathological warfare against agricultural crops (A. Sharma, Gupta, and Devi 2023). The act of plant pathologists studying the dance between our crops and pathogens must start somewhere, and, with cases of disease outbreaks in the field, generally the first practical goal starts with the identification of the pathogen (Ploetz 2009). Indeed, many systems of study within plant pathology have been around for years, if not decades, by now and researchers, of course, have identified the causal pathogens and have often times delved so deep into study, that the question of identification becomes rhetorical. Yet, this study proposes a retraction of questioning to the very baseline of identification; would it be beneficial in terms of piecing a disease management puzzle together if a larger focus was put on determining not only which different bacterial, viral, fungal or other eukaryotic pathogens are present in the field *but also* deeper through to the diversity within each pathogenic entity, the interspecific (different species) or the intraspecific diversity (at subspecies level)? The reasoning behind going as deep as an intraspecific level is that, ecologically speaking, the bigger picture of a pathogen's strategy environmentally and within its host may not be fully elucidated by just looking at a pathogen's species presence alone. The important key here though is that diversification and arrangement of intraspecific variants within farm fields and across agricultural crops may actually play a larger ecological role in that

dance between plant pathogens and their hosts than previously assumed. This raises the question of our ability to achieve the end goal of facilitating better disease management across whichever pathosystem when not considering the comprehensive view of pathogen diversity. The overall goal of this project is to advance our understanding of *X. perforans* pathogen and disease dynamics by begging the examination of whether pathogen intraspecific diversity and its assemblage may play an important role in plant pathogenic interactions, thus greatly contributing not only to how we should develop strategies of managing diseases, but also in the strategic breeding of plants against these pathogens (Timilsina et al. 2020; Ploetz 2009).

The importance of studying pathogen diversity

In conclusion of what was just previously detailed, when dealing with pathogens in the field, one important thing to consider really could be the extent of pathogen diversity present. While possibly more uncommon in agricultural systems, this process reaches close to home with one familiar pathosystem: seasonal influenza (the flu) as an endemic pathogen affecting humans year after year. With seasonal influenza being a nationwide talking point annually, and with the time of year which it is prevalent even being termed the 'flu season', this disease's management practice of choice that eases societal mind is the seasonal flu-vaccine. Importantly though, the flu vaccine is not simply one cure-all that is used over and over every season, but it is formulated compositionally year after year prior to the upcoming flu season (Bandi and Bertsimas 2019). The way researchers are able to do this is by making use of influenza sampling data and reviewing not only which strains are dominant at the given time but also by looking at any low-frequency strains present within the population to predict whether the current presiding strain will remain dominant in circulation or whether other strains may rise in frequency by the following flu season. This review of the pathogenic diversity along with knowledge of those

strains allows them to formulate a vaccine for broad regions but more recently, even regionally specific to increase efficacy (Bandi and Bertsimas 2019; Sambuddha Chakraborty and Chauhan 2023). Reflecting on the constant need to update the vaccine yearly, it is simply a result of the coevolution that is happening between the pathogenic flu and its host, humans. In agricultural systems, what that looks like between a plant and a pathogen is an arms race where the plant is constantly adapting to resist the pathogen and the pathogen is constantly adapting to maintain its presence within the host (not just through increased virulence but broadly through a range of adaptations specific to any given pathosystem). While vaccines for plants really are not the disease management method of choice for practicality reasons (and the fact that plants are not the same as humans), classical plant breeding approaches to find resistance (R) genes against pathogenic species have held up in the past but eventually suffer due to insufficient resistance against all strains of the pathogen and rapid pathogen evolution (Zewdu et al. 2022; Greenwood, Zhang, and Rathjen 2023). With definite room for improvement in the field of breeding for resistance, new approaches have started to make similar use of pathogen survey, much like in the pathosystem of influenza in humans, where genomic sequencing of a large diversity of strains causing disease in crops allows for more rapid identification of resistance genes and a variety of intraspecific pathogenicity factors to use as targets for specific plant genome editing efforts (Amas et al. 2023; Greenwood, Zhang, and Rathjen 2023; Zewdu et al. 2022). With pathogen diversity holding an important role in the coevolutionary arms race with the host, researchers are tasked to answer the major question of whether it is possible to stay ahead of pathogens within their coevolutionary arms races and to predict and negate pathogen success.

The pathosystem: *Xanthomonas perforans* causing bacterial leaf spot on tomato and pepper, an endemic pathogenic species of the Southeast United States

While researchers all over the world dedicate their time to studying various pathosystems, the pathosystem that we work with is *Xanthomonas perforans* causing bacterial spot disease on tomato and pepper plants (Eric A. Newberry et al. 2023). This species was first isolated from Florida tomato fields in 1991 where it quickly prevailed over the previously predominant, closely related *Xanthomonas* species *X. euvesicatoria* before rising to now be considered a globally dominant species(A. P. Hert et al. 2009; E. A. Newberry et al. 2019; Potnis 2021). Bacterial spot is a seedborne disease that causes necrotic lesions on the vegetative parts of the plant and on the fruits where severe infections can cause defoliation of the plant and subsequently yield loss, a major concern for farmers (Abrahamian, Klein-Gordon, et al. 2021). The disease is endemic to most of the tomato/pepper growing regions around the world. To negate these losses caused by this century old disease, selecting optimal plant cultivars is one way to manage the disease. With pepper farming, resistant cultivars are commercially available for use, yet no such resistance has been documented in tomatoes. Although efforts have been made for breeding resistant tomato cultivars, not much success has been had due to a range of issues including the genetic diversity present and variability of effectors within the species which makes breeding for single-gene resistance difficult (Abrahamian, Klein-Gordon, et al. 2021). Considering the lack of a resistant cultivar, tomato farmers have been using chemical applications such as copper-based antibacterials, yet a rise in resistance against copper products urges farmers to use a range of cultural management practices such as the use of certified clean seeds and pathogen-free transplants or the employment of biocontrol strains against *X. perforans* (Abrahamian, Sharma, et al. 2021). Considering that one of the challenges faced in this

pathosystem (on tomato specifically) is that the pathogen's intraspecific diversity complicates classical plant breeding approaches, taking steps to really get an in depth understanding of pathogen population structure may help formulate better ways of managing this endemic disease by elucidating genetic markers as possible targets of modern plant breeding efforts.

Present described intraspecific diversity within *X. perforans*

Within the past 32 years following its first isolation in Alabama, descriptions of the diversity within the species have increased in number and have evolved. Coinciding with an exponential increase in published genomes on NCBI, is an increase in the described diversity of the species (Figure 1-1): starting in 2014 when the first genome was published, the species was described as having 3 ‘groups’, in 2019 the species was described as having 6 different ‘sequence clusters’ using the 142 published isolates, and today with a total of 530 genomes available, 8 different ‘sequence clusters’ are present (Figure 1-2) (Bhandari et al. unpublished data; E. A. Newberry et al. 2019). With the current way of describing this intraspecific diversity being a ‘sequence cluster’ (SC), a sequence cluster simply contains strains that are more closely related to each other than to strains of other SCs. These sequence clusters are characterized using phylogeny based on core single-nucleotide polymorphisms (SNPs) across the published genomes on NCBI with RheirBAPS (Cheng et al. 2013; Tonkin-Hill et al. 2018), as previously described (Newberry et al. 2019).

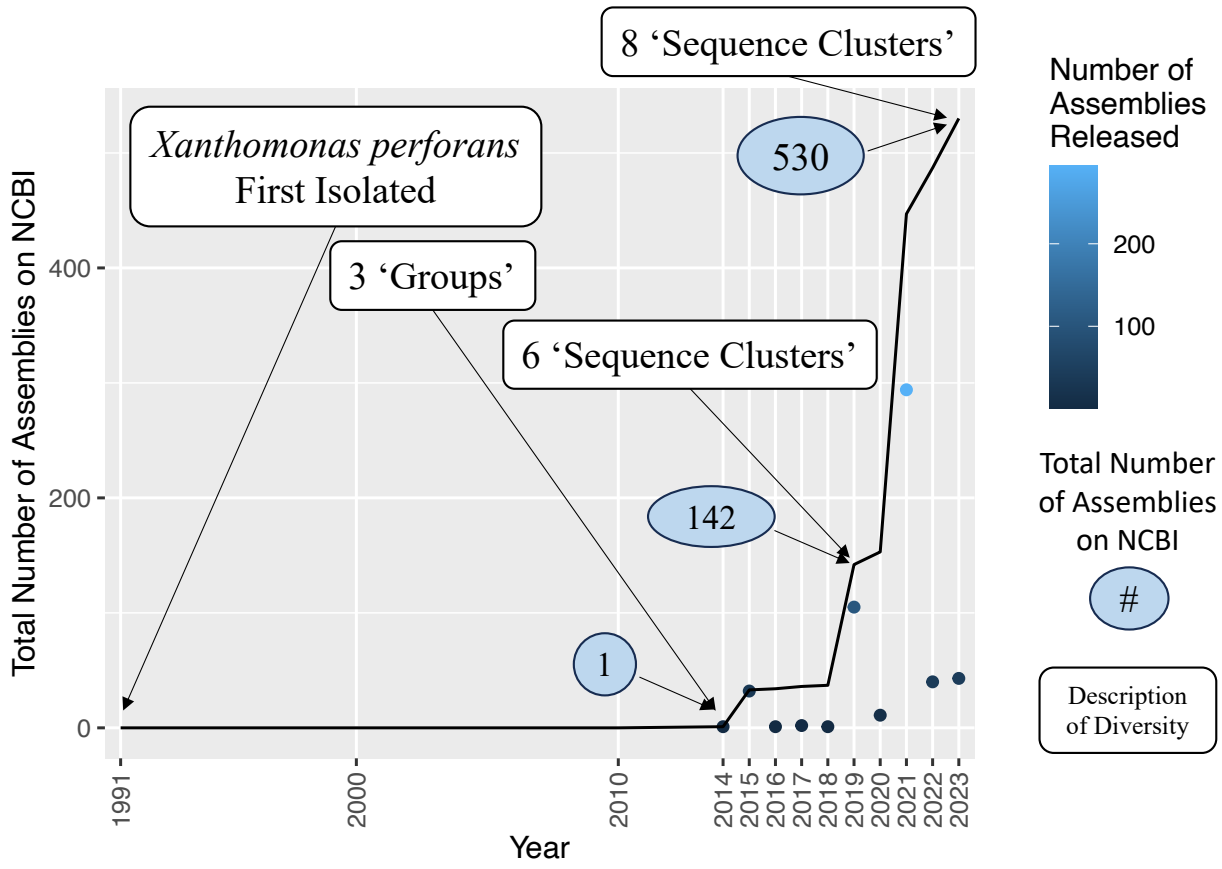

Figure 1-1: Total number of *X. perforans* assemblies on NCBI coincides with species diversification. Following the first isolation of *X. perforans* in 1991, 32 years of research have resulted in a total of 530 assemblies of the species being published to NCBI. Over time, this increase in the amount of published assemblies coincided with an increase in described intraspecific diversity with the species previously being described as 3 'Groups' in 2014, to 6 'Sequence Clusters' in 2019, to a total of 8 'Sequence Clusters' in 2023 (Bhandari et al. unpublished data).

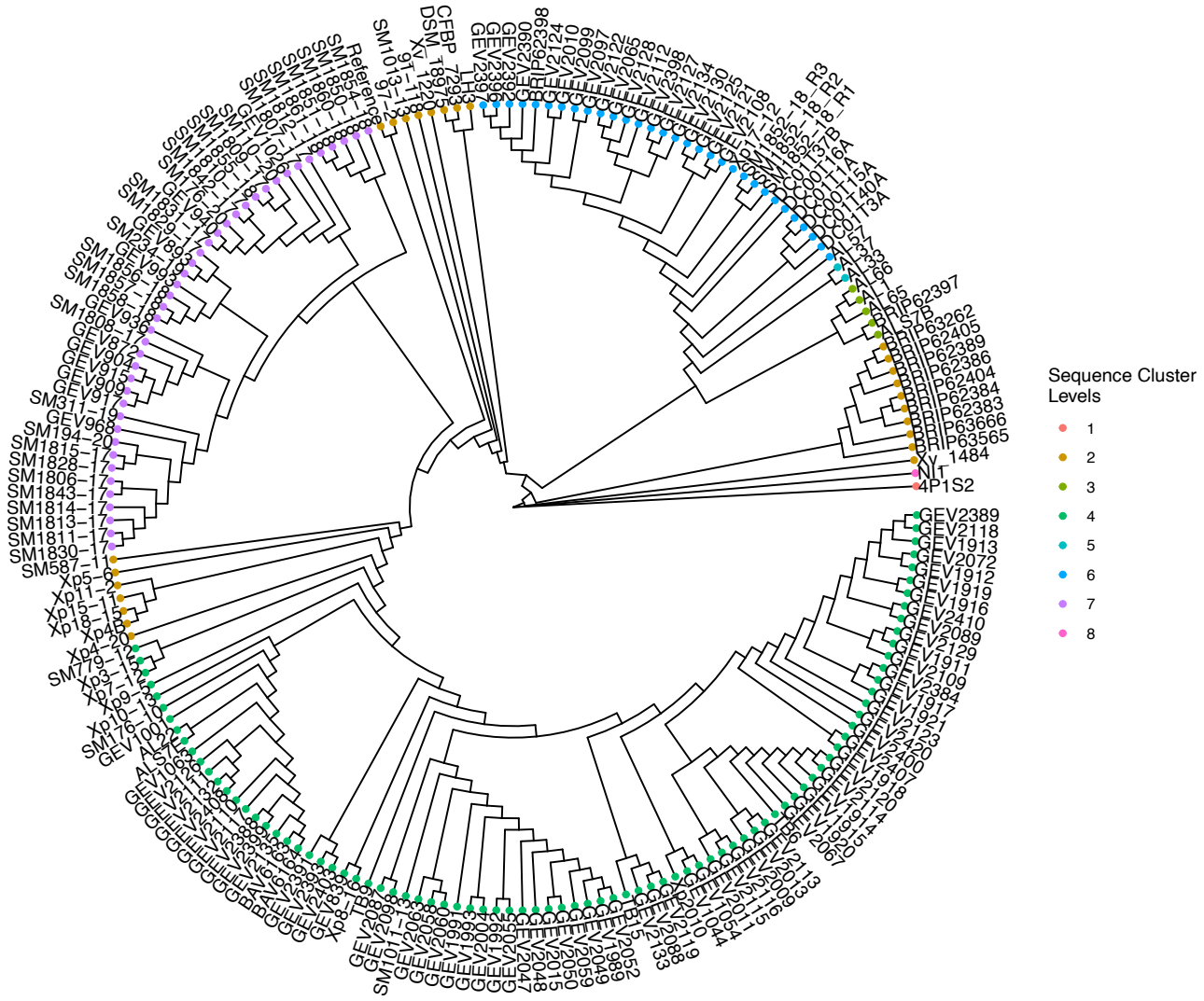

Figure 1-2: Intraspecific diversity of *X. perforans* grouped into sequence clusters (SC). *X. perforans* groups into 8 different sequence clusters when the SNPs in the core genome alignment of 191 RefSeq NCBI *X. perforans* assemblies are compared to each other using RheirBAPS (L. Cheng et al. 2013; Tonkin-Hill et al. 2018). These 'Sequence Cluster Levels' are not indicative of actual sequence cluster names, just of the groups of sequence clusters of which six are named based on prior publication (E. A. Newberry et al. 2019).

Genetic Processes Underlying *X. perforans* Diversification

A precursor to the presence of intraspecific diversity of a species would inevitably be the process of genetic diversification of the lineage. Along with describing the first six SCs, Newberry et al. (2019) also set out to understand patterns of gene flow between *X. perforans* and closely related and what they found was that genome analyses of those 6 SCs did show that recombination events with closely related donors *X. euvesicatoria* strains, *X. euvesicatoria* related pathovars, and species outside of the *X. euvesicatoria* complex, resulted in proportions up to 8% of the core genomes of SCs (E. A. Newberry et al. 2019). Not only did they find that recombination was occurring, but that there were differential rates of recombination with respect to donor lineages across the different SCs where SCs 1-3 had major proportions of their core genome associated with *X. euvesicatoria* as the donor lineage and SCs 4-6 not only recombined with the donor lineage *X. euvesicatoria*, but also *X. euvesicatoria* related pathovars and SCs 5 and 6 had relatively more recombination with donor lineages outside of the *X. euvesicatoria* complex. While this work has not been conducted on a larger set of available genomes since 2019, it is hypothesized that not only has recombination with closely related species along with intraspecific horizontal gene transfer contributed to the diversification of *X. perforans*.

Factors facilitating the genetic diversification of the pathogen

Reflecting on the dance between the pathogen and its host mentioned earlier, studies have shown an intimate link, termed the coevolutionary arms race, between the evolution of a pathogen with its associated virulence factors and the pressures host defenses apply to the pathosystem (L.-S. Ma et al. 2014). This coevolutionary arms race provides a battleground within which natural selection of traits (which were lucky enough to succeed the opponent's advances) drives each species to evolve. Imagining the pathogen-host battleground as a

hypothetical card game, one way each opponent in the arms race could increase their chances on winning is by simply increasing the number, and diversifying the cards they can play (L.-S. Ma et al. 2014; Zaman et al. 2014; Mauricio et al. 2003b). In phytopathosystems such as that of *X. perforans* on tomato and pepper, the selection from the host plant (i.e., resistance) is considered the primary driver evolving their pathogens and what the aforementioned card diversification often looks like for a phytopathogen is the generation of genetic diversity of fitness factors (i.e., virulence genes) (L.-S. Ma et al. 2014). While the host plant does play a major role, environmental and agricultural factors such as production or cultural practices hold intimate connections with pathogen evolution as well. – considering that there are no documented resistance genes in tomato, one goal of this study aims to reflect the contribution of these factors (as an aside note though, the role of the plant host in driving diversification of *X. perforans* cannot be completely ruled out because host susceptibility does vary across genotypes, thus, further study is necessary) (Potnis 2021).

To dive into production practices as a possible driver of diversification, considering the nature of the pathosystem and the fact that *X. perforans* is a seed-borne disease, there are a couple of factors within the tomato production chain that could facilitate the gene flow that Newberry et al. elucidated in 2019 as a method of diversification (E. A. Newberry et al. 2019; Potnis 2021). The end goal of the sale of tomatoes is preceded firstly by the production of tomato seeds in areas anywhere from locally on the farm to intercontinentally – for the pathogen, these seed production areas not only usually have multiple host genotypes (which could harbor closely related species aiding in genetic exchange), but these areas are also environmentally diverse and are essentially one continuous source of pathogen genotypes being introduced into the field (Potnis 2021). Commercially, these seeds should pass through seed certification programs where

highly infected seeds will be removed from production, yet those with low titers of *X. perforans* pass through and are planted and grow in transplant houses before being moved to the field either as pathogen-free or asymptomatic plants. Again, in both the transplant houses and in fields, close contact of plants harboring *X. perforans* may be areas where genetic exchange is occurring for the pathogen; similar to the seed production areas, transplant houses often have multiple host genotypes, and once in the field, pathogenic variants are able to readily mix through dissemination from nearby fields during storms or from alternate host plants/crop debris (Potnis 2021).

Additionally, with consideration of field-level cultural practices as having an influence on pathogen diversity, high disease pressure in the field (exacerbating pathogen evolution through, again, increasing chances for genetic exchange) can easily be achieved through improper knowledge or a simple lack thereof of how to manage this disease when it is present. With previous rampant overuse of copper-based antibacterials already having aroused pathogenic resistance, farmers are encouraged to manage disease by not only using certified clean seeds and pathogen-free transplants, but optimally by reduction of chemical use paired with the selection of optimal tomato varieties and the employment of proper sanitation techniques of removing debris possibly harboring carryover inoculum (Abrahamian, Klein-Gordon, et al. 2021; Potnis et al. 2015).

With the very nature of a seed-borne disease paired with both production and field-level cultural practices possibly facilitating the genetic diversification observed in *X. perforans*, one parameter that always encompasses the pathogen along its journey is the environment. Environmental pressures such as temperature, humidity, and carbon dioxide (CO_2) or ozone (O_3) levels have been known to shape niche ecological niches of organisms, even microbes. Current

global changes such as climate change are placing pressure on species in changing environments to either urgently adapt and evolve to the new parameters and or to constantly migrate and track their original niche (Santini and Ghelardini 2015; Sukumar Chakraborty 2013; Liu et al. 2019). Considering that farm land providing hosts for these phytopathogens is set and does not readily move to maintain a microbe's optimal abiotic niche space, microbial evolution and diversification of environmental fitness factors may be the way to go for microbes such as our pathogenic *X. perforans*.

A pilot study showed that described genetic diversity is present in Alabama farm fields

Regardless of the present genetic diversity or underpinnings of diversification, the question remained as to whether or not this diversity was actually being represented in farm fields. A major predecessor to this work began answering that question by finding that not only is *X. perforans* genetically diverse (E. A. Newberry et al. 2019), but that heterogeneity is also reflected in the field and is represented by the presence of cooccurring SCs within AL farms (E. Newberry et al. 2020). Across four different locations, Newberry et al. (2020) observed that five out of the six sequence clusters described at the time were found to cooccur in fields, proving a preliminary answer but subsequently bringing up a series of questions as to whether or not this heterogeneity was common and how many SCs were normally found cooccurring, whether or not there were seasonal or spatial shifts in these SCs and their abundances relative to each other in the field, and whether or not these pathogen dynamics could explain disease severity outcomes. This study aims to characterize and report the intraspecific diversity within *X. perforans* with hopes of moving this field of research one step closer to winning a seemingly losing battle, and these questions are among those I have tried to approach or advance with my work primarily in my 'Chapter 2' (Objective 1): I address how many sequence clusters are found to cooccur in

farm fields across the Southeast U.S. and whether or not that number can be attributed to disease severity, whether or not there are spatiotemporal shifts in the number of SCs present, and lastly, I study environmental parameters as possible factors facilitating heterogeneity of the pathogen population. For my 'Chapter 3' and 'Chapter 4' (Objective 2), considering that coinfection of pathogens in various systems has been known to increase disease severity of the host, we pondered whether this observation would also apply when coinfection was made up of two closely related strains of *X. perforans*, just in different SCs (Alosaimi et al. 2021; Bellah, Seiler, and Croll 2023a; Kim et al. 2022). To do this however, with the intricacy of studying pathogens so closely related, a protocol for achieving such high resolution into the pathogen population was created in 'Chapter 3'. In 'Chapter 4', I asked the question as to whether disease severity would increase when comparing a monoinfection to an intraspecific coinfection coinfection was answered along with elucidating factors of strain:host and stain:strain interactions within those coinfection dynamics. Lastly, with my 'Chapter 5' (Objective 3), I uncover how coinfecting pathogen populations (two SCs) of *X. perforans* on susceptible and resistant pepper may respond to global changes in the field by using an extreme environmental variable, elevated O_3. At a basal level I sought to understand the driving factors for disease and pathogen dynamics in *X. perforans* on tomato and pepper with these three objectives:

Objective 1: Spatiotemoral Surveillance of the Pathogen Population Across the Southeastern U.S.,

Objective 2: Experimentation with Coinfection and Disease Outcomes in the Greenhouse

Objective 3: Assessment of Pathogen Response to Host Defense and an Extreme Environmental Variable.

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2. CHAPTER TWO

Spatiotemporal Surveillance of the Pathogen Population Across the Southeastern U.S.

Abstract

With *Xanthomonas perforans* having been isolated from and heavily documented in Florida, this study marks a comprehensive sampling of tomato farm fields across four Southeastern U.S. states. We conducted spatial and temporal sampling across Alabama, Georgia, South Carolina, and North Carolina across the growing season and profiled the pathogen population using high resolution metagenome sequencing. We observed that co-existence of different lineages of *X. perforans* is common across the fields and that different lineages predominate in the neighboring states or even neighboring farms in the same state. Spatiotemporal shifts were observed in the pathogen population and trends in these shifts over the span of three years suggest carryover of pathogen populations from one year to the next, or introduction of a new pathogen population from various sources. Apart from profiling of the pathogen population, another objective of this study is to identify factors involved in driving disease dynamics in the fields. Association of climatic parameters with pathogen and disease data suggests that certain abiotic factors could be at play in driving presence and number of pathogen lineages along with aiding in increasing disease severity. Considering that management of bacterial leaf spot caused by *X. perforans* has not been successful thus far, this overview of the pathosystem within farm fields may be useful in the development of new management strategies.

Introduction

Research in the field of plant pathology underpins one of the most important aspects of our society today: agricultural food security. With plant pathogens contributing to the loss of 20-25% of harvested crops worldwide and with food prices on the rise, researchers are fighting with plants in an ongoing battle against quickly-evolving pathogens (Savary et al. 2012; P. S. Teng and MacKenzie 1984; Dixon 2012). In a plant pathosystem, this ongoing battle between a pathogen and its host, termed a 'coevolutionary arms race', often pushes the host plant to employ mechanisms of defense against pathogens who are alongside evolving to evade or overpower those defense mechanisms at the same time (Anderson et al. 2010; Kareiva 1999). With pathogen virulence genes and corresponding plant resistance genes being some of the most variable genes within these plant-pathogen pairs, studies have shown a gene-for-gene link between the two where selection imposed by the host plant can push a pathogenic population to diversify linked virulence genes and vice versa (Karasov, Horton, and Bergelson 2014; W. Ma et al. 2006). In the event that pathogen diversity is already high and can quickly overcome the plant defense response/evolutionary advances or when monocultures are routinely planted, decreasing the ability of the plant to evade the pathogen, long-term coevolution has been shown to break at the plant's expense, often leading to major crop losses (Thompson and Burdon 1992; Fones et al. 2020; Castledine et al. 2022). With this link between the plant and the pathogen being apparent, what would happen in a system where no host resistance genes have been described? Would there still be a push towards diversification within the pathogen?

The pathosystem of *Xanthomonas perforans* causing bacterial leaf spot (BLS) on tomato and pepper is an example of a pathosystem where no such host resistance has been described in tomato. However, over the past 32 years following its isolation in Florida, not only has the

species displaced the previously dominant *Xanthomonas euvesicatoria*, but studies have also shown a rapid change in pathogen genotype and phenotype where sequencing of the species within farm fields routinely discovers more diversity (Klein-Gordon et al. 2021; E. A. Newberry et al. 2019; Bhandari et al. unpublished data). Description of this diversity has been changing since 2014 where the intraspecific diversity was first described as 3 'groups' before being adapted to 6 different 'sequence clusters' (synonymous to pathogen lineages) in this lab in 2016, and further 8 different 'sequence clusters' presently (E. Newberry et al. 2020; Bhandari et al. unpublished data). Describing the intraspecific diversity with this term sequence cluster (SC) is a way of grouping strains within the species into clusters within which the strains are more closely related to each other than to strains of other SCs using core single nucleotide polymorphisms (SNP) across genomes published on NCBI followed by Baysian heirarchial clustering into different lineages using RheirBAPS (L. Cheng et al. 2013; Tonkin-Hill et al. 2018; E. Newberry et al. 2020; Bhandari et al. unpublished data). While the description has been changing to keep up with newfound diversity, with a lack of obvious resistance within tomato to drive this diversification, many questions have been raised surrounding the driving factor aside from the host plant in this diversification (Newberry et al. 2019, Potnis et al. 2021).

Within plant pathosystems, while the host plant has been shown to be a major driving force in the evolution of pathogens, a range of other biotic and abiotic factors all play a part in shaping pathogen and disease dynamics as well. Along with the host either being susceptible or resistant, environmental factors such as temperature and humidity, agricultural practices such as the application of biologicals or chemicals, and inter- and intraspecific interactions with other members of the microbiome or within the same species can drive pathogen diversification and disease dynamics (Figure 2-1) (Potnis 2021). Unfortunately combining all of these factors which

can affect pathogen and disease dynamics in the tomato production chain which conveniently combines seed-production areas, transplant houses, and farm fields, all with variability between environment, agricultural practices, host plants, and other microbes (Potnis 2021; Klein-Gordon et al. 2022). Production complexity with each of these steps aids in not only increasing chances for introduction of inoculum but also increasing opportunities for genetic exchange and diversification, thus, study into the tomato production system has, in part, credited its very structure to the shaping of genetic variation existing within *X. perforans* (Klein-Gordon et al. 2022; Potnis 2021).

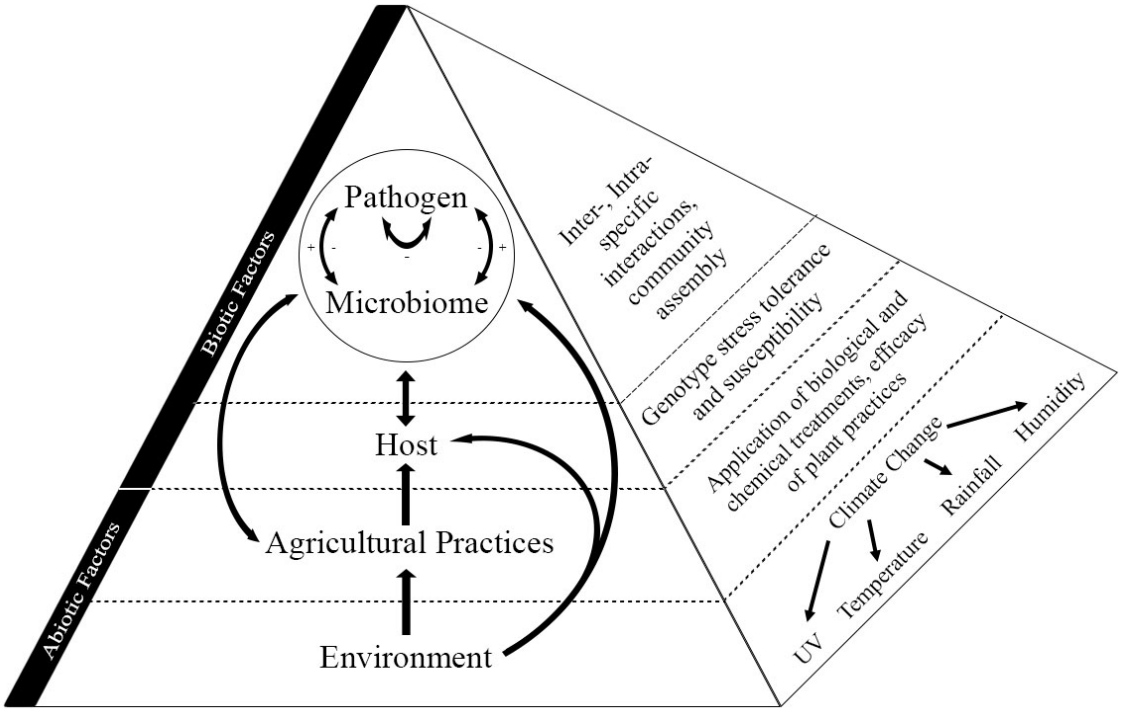

Figure 2-1. Model of biotic and abiotic factors affecting pathogen and disease dynamics within a plant pathosystem.

Issues with the complexity of the tomato production chain are not alone though, current farm management practices have not been shown to successfully reduce the incidence of outbreaks and have even exacerbated the issue in instances where the repeated application of copper-based compounds to control the disease has actually led increased success of the pathogen with the spread of copper resistance (Subedi et al. 2023; E. A. Newberry et al. 2019; Potnis 2021; Bibi et al. 2023). Additionally, ongoing climate change is speculated to change current disease and pathogen dynamics which may disrupt the coevolutionary relationships between hosts and their pathogens leading to pathogen evolution being a more rapid process (Santini and Ghelardini 2015; Sukumar Chakraborty 2013; Liu et al. 2019). A combination of these issues leaves the future for BLS open-ended until further research is done to not only understand underlying factors in diversification of the species, aiding in its success, but also find ways of altering disease management practices that best hinder the success of the pathogen.

With the fact that a baseline understanding of pathogen variation and diversity is essential to understanding factors of disease and potential for adaptation, a recent study sampled tomato fields across Alabama and found, with minimal sampling effort, not only a diverse landscape of *X. perforans* by presence of cooccurring sequence clusters (SCs), but also presence of two novel lineages were isolated that had not been previously identified in other Southeastern states (E. A. Newberry et al. 2019). The study by Newberry et al. (2019) first described six of the 'sequence clusters' that are used in this study, and their observations suggested that there may be an abundance of present diversity essentially just waiting to be sampled. Not only was it important to study this possible undescribed diversity for advancement of basic understanding, but considering that pathogen variation and diversity have been liked to increased virulence and can influence disease outcomes, it may be in growers' best interest that continued efforts are made

towards a better understanding of this pathosystem (Kinnula, Mappes, and Sundberg 2017). To further investigate the extent of pathogen diversity, a pilot study to this project (E. Newberry et al. 2020) developed a strategy of assessing *X. perforans* diversity, including the intraspecific diversity present within the SCs, through a metagenomic approach. This study was conducted to not only understand which lineages were dominant within farm fields across AL, but also to assess whether coinfection by multiple SCs would be observed and found that SCs 3, 4 and 5 were dominant and that cooccurrence of SCs was common among fields (E. Newberry et al. 2020). While questions of SC dominance and cooccurrence were answered, results of this pilot study along with previous research to more questions surrounding pathosystem dynamics in farms outside of the more documented Florida area. (1) Whether this heterogeneity was common, (2) how many SCs were normally found cooccurring, and (3) are there persistent lineages that tend to exist in the fields despite other variable factors of climate or cultural practices? We also wanted to see whether or not spatiotemporal shifts exist in these SCs and their relative abundances in the field, and whether or not pathogen dynamics could be linked to disease severity outcomes. Given the idea that risk factors, particularly climate in this chapter, can influence disease and pathogen dynamics, this chapter aims to characterize and report the intraspecific diversity within *X. perforans* where I address how many sequence clusters are found to co-occur in farm fields across the Southeastern U.S., whether or not that number can be attributed to disease severity, if there are spatiotemporal shifts in the number of SCs present, and lastly, if environmental parameters as possible factors facilitating disease or pathogen dynamics within *X. perforans*.

Methods

Collection and disease severity rating of tomato phyllosphere farm samples with associated metadata

A total of 23 farms across four different states (AL, GA, SC, and NC) of the Southeastern U.S. were represented in this study. Tomato phyllosphere samples were taken during the mid- and end-season and a few during the winter season (GA farms only) across three consecutive years (2020, 2021, and 2022). These samples were collected either by lab members or by extension agents, and along with sampling plant tissue, farm metadata including (but not limited to) parameters such as location of the farm, farm scale (commercial/small), tomato plant cultivar used, and application of chemicals was collected. Upon sample arrival to the lab, disease severity based on typical symptoms of BLS such as the presence of necrotic spots or shot holes was assessed using the Horsfall-Barratt scale (Horsfall 1945). Following disease severity rating, metagenomic DNA from each sample was extracted and sequenced using Illumina (Bhandari et al. unpublished data).

Metagenomic analysis of X. perforans SC relative abundance using StrainEst

Relative abundance of SCs 1-8 within each farm sample was determined using a program called StrainEst which mapped single-nucleotide variants (SNV) within a sample's mixed metagenomic reads to a custom reference SNV profile containing a representative of each sequence cluster mapped to a sequence representative (Albanese and Donati 2017; Bhandari et al. unpublished data). Shannon diversity, an estimation of diversity that considers both species richness and evenness, was then calculated for each sample based on the relative abundance of SCs present (Bhandari et al. unpublished data; DeJong 1975). This work was made possible in part by high

performance computing resources and technical support from the Alabama Supercomputer Authority.

Extraction of NASA POWER data for each farm

NASA POWER data for each farm was obtained from the NASA Langley Research Center POWER Project funded through the NASA Earth Science Directorate Applied Science Program using the "nasapower" package in RStudio (A. Sparks 2018; A. H. Sparks et al. 2023; Posit team 2023). Daily values of parameters including Temperature at 2 Meters (C) (T2M), Dew/Frost Point at 2 Meters (T2MDEW), Wet Bulb Temperature at 2 Meters (T2MWET), Earth Skin Temperature (C) (TS), Temperature at 2 Meters Range (C) (T2M_RANGE), Specific Humidity at 2 Meters (QV2M), Relative Humidity at 2 Meters (%) (RH2M), Precipitation Corrected (mm/day) (PRECTOTCORR), Clear Sky Surface PAR Total (CLRSKY_SFC_PAR_TOT), All Sky Surface PAR Total (ALLSKY_SFC_PAR_TOT), Surface Pressure (kPa) (PS), Wind Speed at 10 Meters (WS10M), Wind Direction at 10 Meters (WD10M), and All Sky Surface Longwave Downward Irradiance (ALLSKY_SCF_LW_DWN) were extracted for the entire season from each farm and further broken down into the average, standard deviation, skewness, and kurtosis for each mid and end season. Descriptions of all these climatic parameters can be found in Table 2-1 of this chapter.

Table 2-1: Climatic parameters extracted to study in association with the pathosystem of *X. perforans*

Parameter	Name	Description
T2M	Temperature at 2 Meters (C)	The average air (dry bulb) temperature at 2 meters above the surface of the earth.
T2MDEW	Dew/Frost Point at 2 Meters	The dew/frost point temperature at 2 meters above the surface of the earth.
T2MWET	Wet Bulb Temperature at 2 Meters	The adiabatic saturation temperature which can be measured by a thermometer covered in a water-soaked cloth over which air is passed at 2 meters above the surface of the earth.
TS	Earth Skin Temperature (C)	The average temperature at the earth's surface.
T2M_RANGE	Temperature at 2 Meters Range (C)	The minimum and maximum hourly air (dry bulb) temperature range at 2 meters above the surface of the earth in the period of interest.
QV2M	Specific Humidity at 2 Meters	The ratio of the mass of water vapor to the total mass of air at 2 meters (kg water/kg total air).

RH2M	Relative Humidity at 2 Meters (%)	The ratio of actual partial pressure of water vapor to the partial pressure at saturation, expressed in percent.
PRECTOTCORR	Precipitation Corrected (mm/day)	The bias corrected average of total precipitation at the surface of the earth in water mass (includes water content in snow).
CLRSKY_SFC_PAR_TOT	Clear Sky Surface PAR Total	The total Photosynthetically Active Radiation (PAR) incident on a horizontal plane at the surface of the earth under clear sky conditions.
ALLSKY_SFC_PAR_TOT	All Sky Surface PAR Total	The total Photosynthetically Active Radiation (PAR) incident on a horizontal plane at the surface of the earth under all sky conditions.
PS	Surface Pressure	The average of surface pressure at the surface of the earth.
WS10M	Wind Speed at 10 Meters	The average of wind speed at 10 meters above the surface of the earth.
WD10M	Wind Direction at 10 Meters	The average of the wind direction at 2 meters above the surface of the earth.

ALLSKY_SCF_LW_DWN	All Sky Surface Longwave Downward Irradiance	The downward thermal infrared irradiance under all sky conditions reaching a horizontal plane the surface of the earth. Also known as Horizontal Infrared Radiation Intensity from Sky.

Data Analysis

The question of whether Shannon diversity, number of sequence clusters present, or the dominant sequence cluster present affects disease severity was addressed using a linear model in RStudio. To answer whether climatic factors could be considered predictors of presence/absence of SCs, a regular linear lasso approach was used to select climatic variables as important variables/predictors of each response variable before a binomial generalized linear model was run on those selected to find any significance (Norris et al. 2006). For generalized linear model studies, predictors with a Pr(>|z|) less than 0.05 ($p<0.05$) were considered to be significant and their impact, either positive or negative, was considered based off of the sign of the estimate (Murtaugh 2014). This regular linear lasso approach was also used to select climatic variables as important predictors of disease severity, number of SCs present, and Shannon diversity before those important predictors were assessed as significant predictors of the response variable using an ordinal logistic regression (Norris et al. 2006). Coefficients were considered as significant if their t value was larger than 2, and their impact was interpreted based on the sign of the coefficient with a positive sign regarding a positive correlation and negative sign thus a negative correlation (Cameron et al. 2014).

Results

Number of SCs co-occurring and dominant SC found in fields were variable across states, and number of SCs was positively associated with disease severity

A total of 23 farms across four states of the Southeastern U.S. were sampled across the years 2020, 2021, and 2022 for metagenomic DNA before high-resolution sequence analysis was conducted to monitor *Xanthomonas perforans* population dynamics. Additionally, disease severity was recorded alongside for each sample. Across all farm samples, there were on average 2.29 SCs present within each sample, with North Carolina farms reaching a maximum of 3 SCs, states Alabama and Georgia reaching a maximum of 4 SCs, and South Carolina reaching the highest number of SCs within a sample: 5 SCs (Figure 2-2.A). Commercial and small scale farm types differed in average number of SCs present with small scale farms having less SCs present on average with 1.67 per farm sampled as opposed to 2.52 per commercial farm sampled in this study (Figure 2-2.A). Regarding dominant SCs present (SC with the highest abundance relative to other SCs in the sample) within each state and farm type, out of a total of 65 farm samples, only 5 had no SCs present based off of analysis with StrainEst (Figure 2-2.B.). Alabama represented farms with dominance of 4 different SCs (SC3, SC4, SC5, and SC6) whereas states South Carolina, Georgia, and North Carolina only represented farm samples with 3, 2, and 1 different SCs being found as dominant, respectively (Figure 2-2.B). With SC3 being dominant across 31 farm samples in total (31/44 = 70.45% of the time it was present), this sequence cluster was sampled as dominant the most in Georgia and South Carolina farm samples, but in Alabama, while SC3 was dominant in some samples, SCs 4 and 6 were primarily the dominant sequence clusters found within farm samples. In North Carolina, SC4 was the only dominant SC found. SCs 4 and 6 were dominant in fields 50% of the time they were present, and SC5 was

dominant in 20% of the fields which it was present. Disease severity was found to be positively correlated with number of SCs present (p=0.005, Kruskal-Wallis) (Figure 2-2.C).

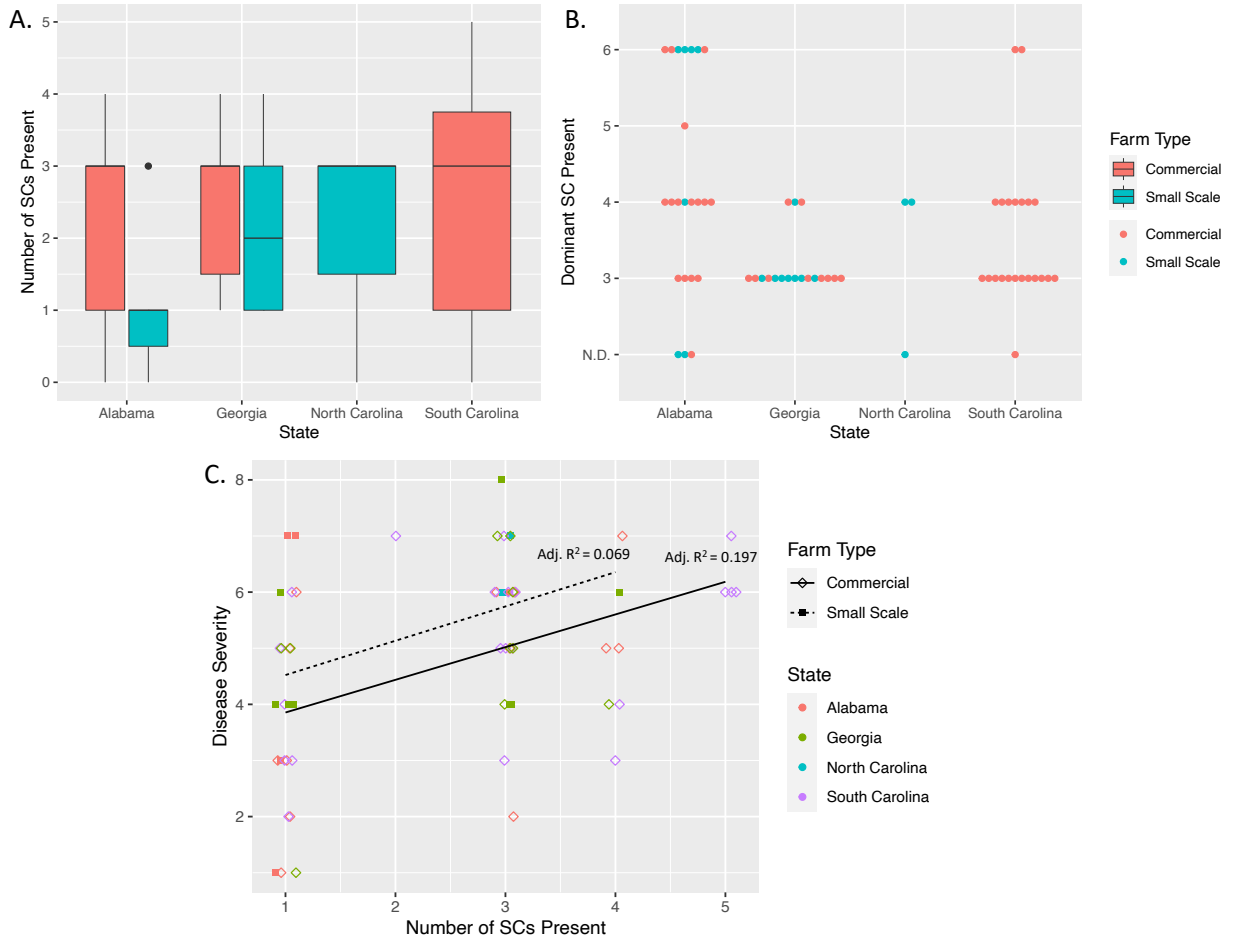

Figure 2-2. An overview of *X. perforans* presence and disease severity in farm fields across the Southeastern U.S. A total of 23 farms across four states of the Southeastern U.S. were sampled across the years 2020, 2021, and 2022 for metagenomic DNA and disease severity to study the pathogen dynamics of X. perforans. (A.) Number of SCs per farm within each state separated by farm type in terms of being either commercial or small scale. (B.) Dominant SC present (SC with the highest abundance relative to other SCs in the sample) within each state and

by farm type. ‘N.D.” denotes that presence of X. perforans SCs was not detected, this was observed in 5 farm samples. (C.) Disease severity of farm samples (with SCs present) by number of SCs present separated by state and farm type with a linear model line showing correlation between the two by farm type.

Number of SCs cooccurring and dominant SC found in fields were not only variable across states, but also over time

Stacked area plots for each farm show intraspecific diversity of *X. perforans* by relative abundance of SCs over time and space within the 13 out of 23 farms that had more than one sample (Figure 2-2). Out of the 61 farm samples from those 13 farms, 38 of those samples had multiple SCs cooccurring among plant tissue (62% of samples). While the dominance of particular SCs, namely SCs 3, 4, 5, and 6 are apparent with the stacked area plot, SCs 1, 2, 7, and 8 are represented in farm samples as well, meaning that all eight SCs described have been represented in this sampling. Presence and dominance of SCs was observed changing over time in some farms, yet in others, pathogen dynamics seemed to remain relatively consistent based on relative abundance over time. To characterize temporal pathogen dynamics in those farms sampled more than once starting with Alabama, while two farms (Farm 2 and Farm 4) show some semblance of consistency across SC relative abundance over time, heterogeneity within those farms decreases by the last sample in each. In general with these Alabama farms, a lot of variability is seen amongst the SCs present and their relative abundances, for example: in Farm 1 (a small scale farm), the dominant SC completely changes from one year to the next, and not only do Farms 2 and 4 decrease in their heterogeneity over time, Farm 3 started out with almost equal populations of SC3 and SC4 before flipping between the two over time. In Georgia farms,

SC 3 was primarily dominant and SC4 often present alongside it at lower abundances. Pathogen dynamics within these farms do not appear very variable across space or time with both small scale and commercial farms being represented. With SC3 dominating across most of the farms, not much intraspecific heterogeneity was observed in terms of equal relative abundances. In South Carolina farms sampled more than once, SC 4 and SC3 were primarily dominant with rapid changes being observed in two of the farms (Farms 8 and 10), all three were commercial, where SC4 relative abundance seemed to change readily. In the remaining Farm 9, pathogen dynamics were consistent with SC3 being dominant and there being less heterogeneity. Lastly, in the one North Carolina farm sampled more than once, SC4 was dominant, yet this farm also had a respectable relative abundance of SC2 which, in contrast, held very low relative abundances across other fields. Temporally from mid to end season for this farm in 2020, not much change occurred.

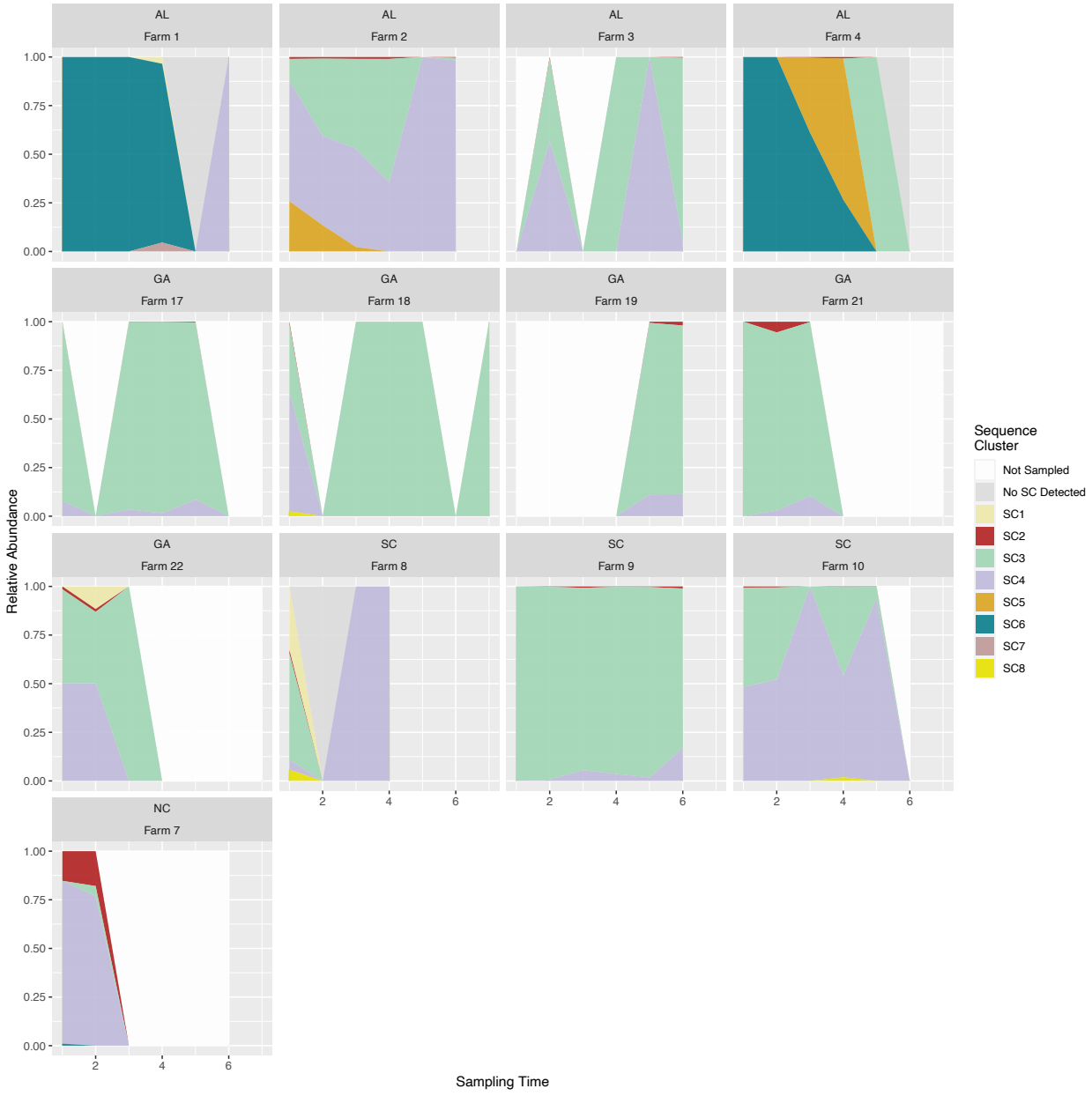

Figure 2-3. The extent of farm sampling across 13 farms that had >1 sample, with 6-7 different sampling times over three years profiling *X. perforans* relative abundance of SCs 1-8. Stacked area plot showing relative abundance of SCs 1-8 across 13 farms (which must have had more than one sampling time to create a plot) within samples taken across three years and 6-7 different sampling times. Sampling times 1-6 denote mid-season then end-season sampling for 2020, then 2021, and 2022 respectively except in farms 17, 18, 21, and 22, where sampling time

1 and 2 are similar to the other farms, yet 3 is an addition of sampling from the winter season of 2020, and then 4-7 represent mid-season and end-season for 2021 and 2022 consecutively. Farm states Alabama, Georgia, South Carolina, and North Carolina are represented and marked above farm name, and for reference, Farms 1, 7, 17, and 18 were small scale farms and the rest were commercial farms.

Disease and pathogen dynamics are associated with metrics of four different climatic parameters used in this study

Using a regular linear lasso approach to select climatic parameters as important predictors of presence/absence of SCs, number of SCs present, Shannon diversity, or disease severity of farm samples before either a generalized linear or an ordinal logistic regression was run to determine significance, metrics (average, standard deviation, kurtosis, skewness) of four different climatic parameters were selected as significant (p<0.05 or |t value|>2): all sky surface photosynthetically active radiation (PAR) total, wind direction at 10 meters, average precipitation corrected (mm/day), and temperature at 2 meters range (Tables 2-2 and 2-3).

Considering climatic factors as predictors of presence/absence of SCs, statistics could not be run for SC7 considering there was only one observation, and no climatic parameters were selected as important predictors for SC2. For SCs 1, 5, 6, and 8, climatic parameters were selected as important predictors, yet none were significant. For SCs 3 and 4, however, regarding presence/absence of SC3 and its association with climatic parameters, climatic parameters were selected as important predictors with standard deviation of both all sky surface PAR total and wind direction at 10 meters being considered two statistically significant important predictors (p=0.016 and p=0.026, respectively) with standard deviation of all sky surface PAR total

negatively effecting presence of SC3 and standard deviation of wind direction at 10 meters positively affecting SC3 presence (Table 2-2). For SC4 presence/absence, the climatic parameter average precipitation corrected (mm/day) was selected as the only important predictor and was considered to be statistically significant (p=0.017) and negatively effecting SC4 presence (Table 2-2).

Table 2-2: Climatic variables as predictors of SC relative abundance (significant values only)

Model	Response	Predictor	Estimate	Std. Error	z value	Pr(>|z|)
Generalized Linear, Binomial	SC3 Presence/Absence	Standard Deviation of All Sky Surface Photosynthetically Active Radiation (PAR) Total	-0.28093	0.11673	-2.407	0.0161
Generalized Linear, Binomial	SC3 Presence/Absence	Standard Deviation of Wind Direction at 10 Meters	0.13556	0.06102	2.222	0.0263
Generalized Linear, Binomial	SC4 Presence/Absence	Average Precipitation Corrected (mm/day)	-0.5895	0.2465	-2.392	0.01676

Considering climatic factors as important predictors of disease severity, number of SCs present, and Shannon diversity, no climatic parameters were selected as important predictors for Shannon diversity, yet parameters were selected and considered significant for number of SCs present and disease severity. For number of SCs present, standard deviation of both temperature at 2 meters range and wind direction at 10 meters along with kurtosis of all sky surface PAR total were selected as important parameters and were considered to also be significant (t value = -3.4, t value = 3.9, and t value = -2.2, respectively) with standard deviation of temperature at 2 meters range and kurtosis of all sky surface PAR total both negatively effecting the number of SCs and standard deviation of wind direction at 10 meters positively effecting the number of SCs (Table 2-3). Disease severity was found to be significantly positively associated with standard deviation of wind direction at 10 meters (t value = 2.4), and changes in disease severity from mid-season to end-season were significantly positively associated with changes in skewness of wind direction at 10 meters (t value = 2.64) from mid-season to end-season (Table 2-3).

Table 2-3: Climatic variables as predictors of pathogen heterogeneity and disease severity (significant values only)

Model	Response	Predictor	Value	Std. Error	t value
Ordinal Regression	Number of Sequence Clusters	Standard Deviation of Temperature at 2 Meters Range	-2.3265	0.68388	-3.402

Ordinal Regression	Number of Sequence Clusters	Standard Deviation of Wind Direction at 10 Meters	0.1846	0.04742	3.8937
Ordinal Regression	Number of Sequence Clusters	Kurtosis of All Sky Surface Photosynthetically Active Radiation (PAR) Total	-0.625	0.28044	-2.2286
Ordinal Regression	Disease Severity	Standard Deviation of Wind Direction at 10 Meters	0.07836	0.03244	2.416
Ordinal Regression	Change in Disease Severity from Mid-season to End-season	Change in Skewness of Wind Direction at 10 Meters from Mid-season to End-season	3.606	1.366	2.64

Discussion

To gain a better understanding of risk factors affecting disease and pathogen dynamics, this study sampled a total of 23 farms across the Southeastern U.S. by assessing disease severity and sequencing metagenomic DNA from tomato leaf material sampled during the mid and end of

growing seasons (a couple of samples were also taken during the winter season). This data allowed us to address intraspecific diversity of *X. perforans* on tomato by profiling which SCs were present in fields, existing either alone or cooccurring with other SCs, and whether their relative abundance was spatiotemporally variable. Additionally, we found if disease severity of these samples could be attributed to the number of SCs cooccurring within samples, and whether or not possible climatic variables were factors facilitating these disease and pathogen dynamics.

Firstly, we found that the number of SCs cooccurring and dominant SC within farm samples was variable across the four states sampled, and that the number of SCs present in a farm sample was positively associated with disease severity. There were on average 2.29 SCs present in farm field samples with this study observing a maximum of 5 cooccurring SCs in South Carolina (Figure 2-2.A). Also, we observed that, on average, our small scale farms had less pathogen heterogeneity in terms of number of SCs cooccurring than commercial farms. Although it could be possible that the slight difference in the number of small scale versus commercial farms sampled could have led to this discrepancy, another hypothesis as to why we are seeing a slight increase in the number of SCs cooccurring in commercial farms is that, in the case that the commercial farms have planted large monocultures of tomatoes, the homogeneity within the host population may be aiding in the heterogeneity of the pathogen population. It has been proposed that monoculture crops could in some way promote pathogen heterogeneity by reducing obstacles of transmission both spatially within the field and genetically due to the lack of host diversity; not only could this exacerbate disease severity but it could also lead to the evolution of pathogens into more virulent strains (Wuest, Peter, and Niklaus 2021; Brown 2015; Cheatham et al. 2009). If small scale farmers generally grow varieties of crops at the same time, including host and non-host genotypes of *X. perforans*, the host heterogeneity could aid in the

reduction of the number of SCs present, and the non-host genotypes could additionally act as physical barriers to the spread of the pathogen (Wuest, Peter, and Niklaus 2021). In fact, regarding disease severity sampled within this study, the number of SCs present was positively correlated to disease severity (Figure 2-2.C). If a maintenance of heterogeneity within these field samples is linked to monocultures of crops, this finding could support the push of farm management practices away from monoculture crops if the host genotypes and other crops planted had been transparent between growers and us as researchers. Regardless, this finding is similar to that of many other studies which have found that coinfection can lead to higher disease severity (Abdullah et al. 2017; Susi et al. 2015; Sadhukhan, Jacques, and Potnis 2023). Focusing now on the SCs found to be dominant in farm samples, out of the four different SCs which were found to have been dominant in at least one sample across the entire study (SCs 3, 4, 5, and 6), one interesting thing to note is that Alabama represented all four of them while South Carolina, Georgia, and North Carolina represented only 3, 2, and 1 different SCs being found dominant. Aside from the fact that Alabama is actually one of the most biologically diverse states (4th highest in terms of species diversity) due to its climatic and geological diversity combined with a 'rich evolutionary past' (Duncan and Wilson 2013), it is possible that Alabama's proximity to the origin of *X. perforans*, Florida, could have something to do with this finding (Figure 2-2.B). A study by Timilsina et al. (2019) indicated that rapid genomic evolution was happening within the *X. perforans* population in Florida with recombination with *X. euvesicatoria*, previously the only *Xanthomonas* species known to Florida, aiding in this diversification (Timilsina et al. 2019). Another study conducted by Newberry et al. (2019) found that proportions up to almost 10% of the core genomes of *X. perforans* sequence clusters in Alabama farm fields were also from

recombination with closely related *X. euvesicatoria*, making it possible that Florida and Alabama are hotspots for *X. perforans* which could explain our observation (E. A. Newberry et al. 2019).

In visualizing the diversity of the *X. perforans* within the farm samples through SC relative abundance over time, it is apparent that cooccurrence is common (62% of the time) and that not only are there spatial shifts in the number of SCs and dominant SC present, but there are also changes in the pathogen population over time (Figure 2-3). Within some of the farms, obvious trends are seen over time with SC relative abundances remaining the same, or some increasing or decreasing at the detriment of other SCs. The pathogen population within these farms could be linked from year to year through carryover of inoculum on seeds, overwintering of inoculum on debris or weeds, or even a maintenance of inoculum at the seed or transplant source for these farms (Abrahamian, Klein-Gordon, et al. 2021). On the other hand, however, more drastically than subtle shifts in SC relative abundances, presence of SCs changes completely in some farms from year to year suggesting that farms could be instead accessing different pools of *X. perforans* diversity through various sources on seed or transplants that they could be introducing to their farms over time (Abrahamian, Klein-Gordon, et al. 2021). Aside from source of inoculum, there could still be selection of SCs environmentally and across the season by environmental factors and various agricultural practices following introduction into farm fields.

Focusing on climatic variables (Table 1) as drivers of not only pathogen dynamics but disease dynamics as well, this study found that disease severity, presence/absence of SCs 3 and 4, and number of SCs were associated with metrics of climatic parameters with a total of four being significant drivers: precipitation corrected (mm/day), temperature at 2 meters range, all sky surface photosynthetically active radiation (PAR) total, and wind direction at 10 meters (Tables

2-2 and 2-3). To go by each climatic parameter starting with precipitation corrected (mm/day) of which a higher average precipitation was negatively associated with SC4 presence, *X. perforans* is considered to disseminate within fields mainly through wind-driven rain and studies associating precipitation with phytopathogen presence and abundance have shown precipitation to increase the abundance of fungal plant pathogens (Q.-L. Chen et al. 2021; Bhattarai et al. 2017; Abrahamian, Klein-Gordon, et al. 2021). It is not directly clear why increased precipitation may lead to decreased presence of SC4, however if the pathogen load on the plant is increased due to higher precipitation levels, the decrease in the presence of SC4 may be due to increased competition with other phyllosphere members. For temperature at 2 meters range of which a higher standard deviation decreased the number of SCs present, with general environmental optima existing not only for plant to function but also for pathogen virulence, one possible reason for this association could be that that variation in the temperature is selecting for specific SCs which are more tolerant to temperature fluctuation (Velásquez, Castroverde, and He 2018a). A study conducted in Brazil on four different pathogenic species of *Xanthomonas* found that temperature had in important role in determining which species are present, and if global changes are causing an increased standard deviation in temperature with extremes being introduced, this could be leading to a reduction in the number of SCs present where only those fit persist (Araújo et al. 2011). The standard deviation of all sky surface photosynthetically active radiation (PAR = light of wavelengths 400-700 nm which is portion of the light spectrum photosynthesized by plants) total was found to be negatively associated with SC3 presence, and a higher kurtosis of this climatic parameter was negatively associated with number of SCs present (Carruthers et al. 2001). Studies in peas have shown that levels of PAR were linked to stomatal apertures through a concentration of zeaxanthin in guard cells, so it is possible that higher

variation in stomatal aperture is associated with a higher number of SCs yet a lower presence of SC3, possibly because of the presence of more SCs yet more study would need to be done to really understand this relationship (Srivastava and Zeiger 1995). For the climatic parameter wind direction at 10 meters, standard deviation was positively associated with SC3 abundance, disease severity of farm samples, and number of SCs present. To understand more about this climatic parameter, wind direction describes the direction specifically from which the wind originates and is measured by degrees on a scale of 0-360 with wind coming from the north lying at 0 or 360 degrees. On average, the farms sampled had wind directions of ~150-200□with winds ranging from the southeast through the southwest and blowing northwest and northeast respectively, meaning that a higher standard deviation would generally consist of winds varying in direction but generally pushing from south to north. We also found that positive changes in skewness of wind direction at 10 meters from mid- to end-season was positively associated with changes in disease severity from mid- to end-season. A positive change in skewness over the season in wind direction with skewness values beginning as negative would push the majority of wind direction values more towards the average and towards a direction south to north direction. With this south-north facing wind direction in mind, many studies have been published on the dissemination of plant pathogens along a south-north disease path (especially rust fungi) (Keane and Brown 1997; Singh, Karisto, and Croll 2021; Böer et al. 2013). In *X. perforans*, though, considering that the southmost state below all of our Southeastern U.S. states sampled is Florida, the origin of the species' first account and one of the major sites of recombination with *X. euvesicatoria* (the species which SC3 had almost 8% of its core genome linked to recombination with) it is possible that wind direction is a major driver in the dissemination of *X. perforans* up from Florida both increasing abundance of SCs such as SC3 and the number of SCs present in

our farm fields, subsequently facilitating disease and increasing severity (E. A. Newberry et al. 2019).

Overall, this study set out to provide an assessment of general pathogen and disease dynamics across the Southeastern U.S. and to address the idea that risk factors, particularly climate in this chapter, are possible influencers of those dynamics. This project achieved those goals and was able to answer questions probed by the pilot study of Newberry et al. (2020) with hopes of providing a broader understanding of *X. perforans* diversity to aid in the development of better management practices.

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3. CHAPTER THREE

Note: this chapter is a manuscript in progress that we plan to submit before the end of this year. My contributions are that I wrote this bioprotocol and set up the experiments, yet I was provided comments for writing by Drs. Gillespie, Watanabe, and Potnis, and I was assisted by Amanpreet Kaur in experiments. Considering that this will be submitted before graduation, I am formatting the following chapter in the way of submission to the journal.

The Use of Flow Cytometry for High Resolution Bacterial Studies *in planta*

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Abstract

Quantifying bacterial populations *in planta* is often an important part of research implemented by scientists studying anything from phytopathogens to the plant growth promoting (PGP) microbiota. With current techniques generally including isolating bacteria from plant tissue and plating onto selective media, moving steps away from culture-based methods could eliminate possible bacterial selection biases on the plate allowing for high-resolution studies into the pathogen population. This is specifically true for the studies involving tracking of multiple pathogens or microbes simultaneously, given that direct interactions among these co-occurring taxa may influence the outcome in culture-dependent methods. Granted the integral nuance and difficulty experienced aiming for such high-resolution, we developed a culture-independent

approach using flow cytometry to allow enumeration of two closely related, co-occurring pathogens *in planta*. Following fluorescent tagging of pathogens as necessary for use with flow cytometry, the streamlined process uses a sonicator to dislodge microbes from phyllosphere tissue which are then processed for input into the flow cytometer machine effectively acquiring absolute bacterial abundance without the limitations of culture-based methods while saving both time and resources.

Key features

- Analysis of closely related bacterial strains from an *in planta* study using flow cytometry, such as coinfection dynamics studies
- Efficacy of using Flow Cytometry for *in planta* studies and comparison to a culture-based approach
- Optimized for the study of phyllosphere microbiota

Keywords: Flow Cytometry, Coinfection, High-resolution Population Analysis, *Xanthomonas perforans*, Culture-independent Phyllosphere Isolation, Sonication

Graphical overview

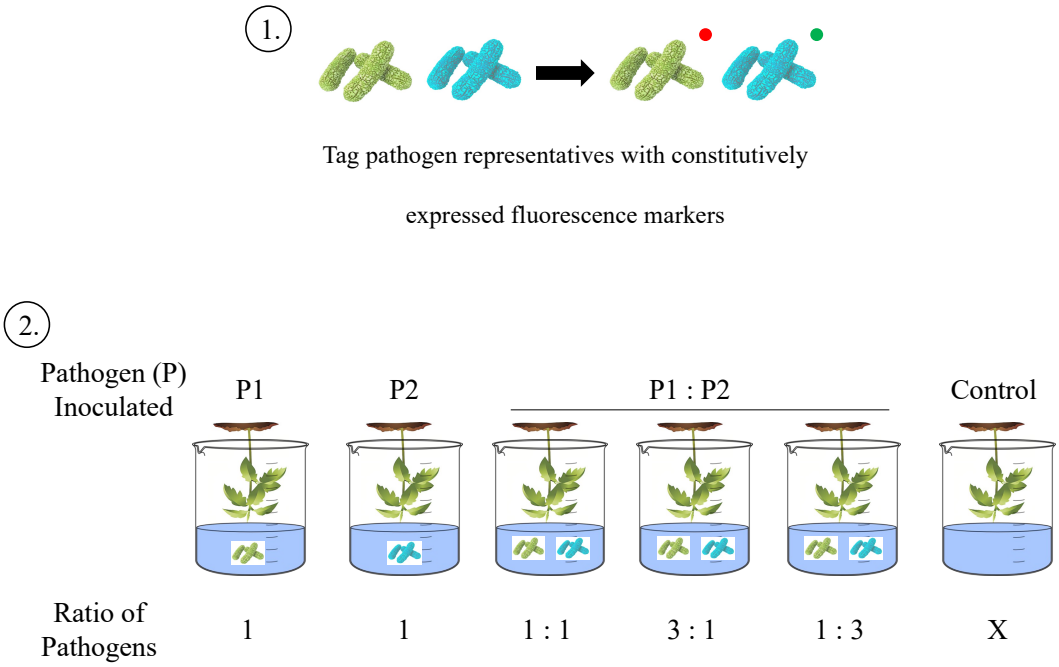


Figure 3-1: Infecting Plants With Fluorescently Tagged Bacteria by Dip Inoculation

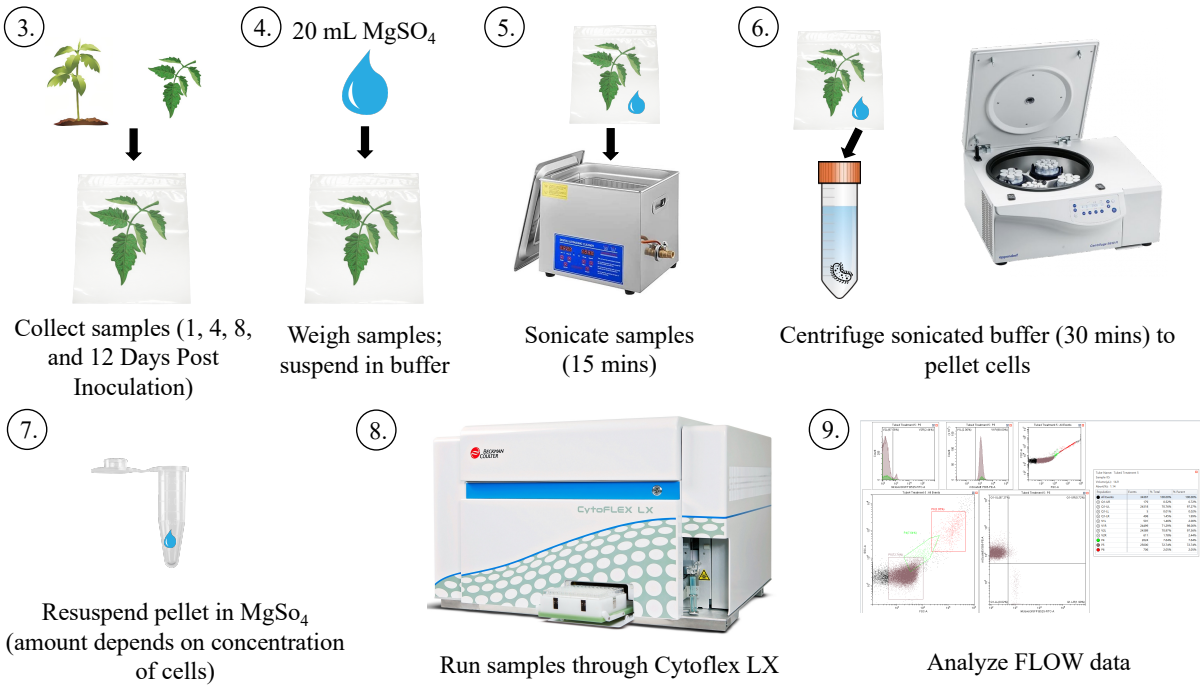


Figure 3-2: Cell Collection from Plant Tissue and Use of Flow Cytometry

Background

This study utilizes coinfection of two closely related strains of the phytopathogen *Xanthomonas perforans* on tomato where, considering the similarity of the strains and the difficulty in distinguishing them physically without distinct markers, high-resolution studies are necessary to understand the population dynamics of each strain simultaneously. When working with multiple bacterial strains tagged with antibiotic markers, use of culture-based methods can introduce biases in the event that antagonistic strain:strain interactions such as the production of bacteriocins against co-plated strains or nutrient limitations towards one strain or another exist (Davis 2014; J. Li et al. 2017; Z. Li, Li, and Bian 2016; Quambusch et al. 2014). Upon question of integrity, such rigorous study would need to be done to justify the absence of such biases that it may be beneficial to shy away from these methods depending on the circumstance (also, see "General Notes: 2").

Although the resolution issue could be solved by simply studying mono-infections of pathogens, previous research has shown that pathogen coinfection is common in natural and agricultural settings with not only different species of pathogens, but also even different strains of the same species (Abdullah et al. 2017; E. Newberry et al. 2020; Sadhukhan, Jacques, and Potnis 2023; Susi et al. 2015). When assessing intraspecific (within species) diversity of *X. perforans* in farm fields, recent studies have found certain strains of the species to be dominant (with dominance of strains being spatiotemporally different), and that heterogeneity is maintained within the farm samples at intraspecific level (Bhandari et al. unpublished data; E. Newberry et al. 2020). Reflecting on this observation with an ecological understanding highlights the fact that maintaining genetic heterogeneity can benefit a pathogen population in the event that shifts in fitness factors beg for change; considering this, assessing intraspecific diversity may give insight

into the way pathogens respond to changes (Arnoldini et al. 2014; Stewart and Cookson 2012; Weigel and Dersch 2018). To gain deeper understanding the intricacies of these ecological dynamics and its feedback into pathogen evolution during within-host colonization, coinfection studies involving population dynamics of individual pathogen lineages and their influence on overall disease outcome are necessary.

Currently, the dominant culture-independent method of sequencing provides a great option to researchers familiar with post-sequencing processing and when monetarily an option, another option is the use of flow cytometry which not only provides an option to study live cells (that must be fluorescently tagged), which sequencing cannot, but along with numerating those live cells, enables researchers to collect data on cell morphology in size and shape as well (Adan et al. 2017; Collier 2000). In this study, we developed a model system to coinfect plants with two strains of *X. perforans* which had different strain relative abundances in the field: one strain was often found at low relative abundance whereas the other strain was often found at high relative abundance (Bhandari et al. unpublished data). The goal here was to use, develop, and optimize a rapid protocol to understand how the population structure of each strain would change when coinfected with another closely related strain at different ratios *in planta*. This protocol presents an overview for the use of flow cytometry to gain high-resolution population dynamics in an *in planta* system of two closely related fluorescently tagged bacteria (Schlechter et al. 2018); while successful in studying a phyllosphere pathogen on tomato, the methodology used in this experiment could be broadly applied to a range of other *in planta* microbial studies.

Materials and reagents

Biological materials

1. *Xanthomonas perforans* strain: Xp 5-6
2. *Xanthomonas perforans* strain: AL65
3. Hybrid Tomato Florida 47 R (Bayer Seminis)

Solutions

1. 0.01 M Magnesium Sulfate ($MgSO_4$) Buffer (see Recipes)

Recipes

1. Magnesium Sulfate ($MgSO_4$) Buffer (0.01 M, 1000 mL)

Reagent	Final concentration	Quantity or Volume
Magnesium sulfate heptahydrate	n/a	2.4648 g
Deionized (DI) H_2O	n/a	1000 mL
Total	0.01 moles/L	1000 mL

Laboratory supplies

1. 1.7 ml microcentrifuge tubes (VWR, catalog number: 87003-294)
2. 100 – 1250 µl universal pipette tips (VWR, catalog number: 76323-456)
3. 150 ml 0.22 µm complete filtration unit vacuum filtration system (VWR, catalog number: 76010-374)

4. 15 ml ultra high performance centrifuge tubes (VWR, catalog number: 21008-216)
5. 25 ml disposable serological pipettes (VWR, catalog number: 75816-090)
6. Sandwich bags (Ziploc)
7. 70 µm cell strainers (Millipore Sigma, catalog number: BAH136800070)
8. Difco™ nutrient agar (VWR, catalog number: 90000-744)
9. Disposable petri dishes (VWR, catalog number: 25384-302)
10. 70 µm Flowmi® cell strainers for 1000 µl pipette tips (Millipore Sigma, catalog number: BAH136800070)
11. Magnesium sulfate heptahydrate ACS $MgSO_4.7H_2O$ (VWR, catalog number: 0662-500G)
12. Silwet® L-77 (PhytoTech Labs, Product ID: S7777)

Equipment

1. Centrifuge 5810R (Eppendorf)
2. CytoFLEX LX N3-V5-B3-Y5-R3-I2 Flow Cytometer (21 Detectors, 6 Lasers) (Beckman Coulter, product number: C40312)
3. Ultrasonic cleaner machine 60A AC100-120V50Hz
4. Forced Air Microbiological Incubator (VWR)

Software and datasets

1. CytExpert Software 2.1

Procedure

A. Pre-Experiment Optimization of Fluorescence and Flow Cytometry Gating

1. For optimal use of this protocol, transform non-fluorescent bacterial strains of interest with fluorescent markers distinguishable from each other when analyzed by flow cytometry. Optimally, transform bacteria with fluorescent markers that are chromosomally integrated to ensure that they will not be lost from the cells during experimentation.

   Note: Two different fluorophores were used in this experiment: mScarlet-I and sGFP2 encoding for red and green fluorescent proteins, respectively. Our bacterial isolates were transformed by electroporation using Tn7 transposon delivery plasmids to express these fluorophores; post-transformation they were selected based on resistance to the antibiotic associated with the fluorophore (Schlechter et al. 2018). In this experiment, the flow cytometer (CytoFLEX LX) was configured so that mScarlet-I was detected on the fluorescent channel 585/42 BP named "PE" and sGFP2 was detected on the fluorescent channel 525/40 BP named "FITC". Both fluorescent probes were excited by the 488-nm laser.

2. To optimize a gating strategy to distinguish fluorescent proteins expressed by each of the transformed bacteria based on fluorescent intensity, run pure cultures of each fluorescent bacteria through the flow cytometer and create gates based on fluorescent intensity of each bacterial population that needs to be identified during the experiment. Again, these fluorescent proteins should be distinguishable from each other and appropriately compensated for spill-over of fluorescence signal into other channels.
3. For optimizing forward (FSC) and side scatter (SSC) of bacterial populations, which represent the size and complexity/granularity of the cell respectively, run pure cultures of

both non-fluorescent and fluorescent bacteria through the flow cytometer and create a gate based on the physical characteristics of the bacterial cells. For *in planta* studies prior to experimentation, it is beneficial to use this step to see where inoculated bacterial populations are observed in relation to phyllosphere microbes present and to note whether or not the size of the inoculated bacteria are similar to that of pure cultures, if not, a new FSC/SSC gate should be created based on the physical properties of the bacterial strains *in planta*. Additionally, if distinguishable, gate the resident phyllosphere microbes as separate from the inoculated bacteria.

4. Following gating, thresholds can also be set to select for only necessary signal data. If desired, set fluorescent intensity for each fluorescent channel used to only show flow cytometry events that meet or exceed that threshold.

   Note: To increase the sensitivity of the cytometer to detect the fluorescently labeled bacterial cells, default thresholds were adjusted to trigger on fluorescent events with an intensity of 1000 units on either the PE or FITC channels. Triggering on these events significantly decreases the number of background events analyzed by the cytometer.

B. Infecting Plants by Dip Inoculation

1. For general use of this protocol to study bacterial population growth within a pathosystem of interest, streak fluorescently tagged bacterial isolates onto respective nutrient rich agar plates and incubate at proper culture temperature and for a sufficient amount of time to prepare a starting inoculum. For this experiment, two strains of fluorescently labeled *Xanthomonas perforans* (as described in the note below) were streaked from glycerol stocks onto nutrient agar plates (NA) supplemented with respective antibiotics and

incubated at 28 °C for two days.

Note: Two strains of Xanthomonas perforans, AL65 and Xp 5-6, were used in this experiment: strain AL65 was transformed using pMRE-Tn7-152 to confer bacterial cells with a constitutively expressed fluorescent protein gene, sGFP2, and resistance to kanamycin; likewise, strain Xp 5-6 was transformed using pMRE-Tn7-145 to express m-Scarlet-I and gentamycin resistance (Schlechter et al. 2018).

See "General Notes: 1" for more information regarding fluorescently tagging bacteria to be used for Flow Cytometry.

2. Concisely, for dip inoculating plants at a level of 10^6 cfu/ml, prepare a solution of 10^8 cfu/ml of bacteria in 0.01 M $MgSO_4$ before diluting 100x into a solution of 0.01 M $MgSO_4$ supplemented with 0.0025% Silwet L-77 (v/v) at a volume suitable to immerse the entire phyllosphere portion of the plant. To prepare inoculum of a monostrain treatment such as this experiment's treatment 'AL65', for example, 8 ml of 10^8 cfu/ml AL65 diluted in 0.01 M $MgSO_4$ was supplemented with 792 ml 0.01 M $MgSO_4$ and 20 µl of Silwet L-77 to obtain a final inoculum of 10^6 cfu/ml (Table 1). For multistrain treatments, which may have similar or different ratios of the bacteria depending on what is necessary for the study, such as this experiment's treatment '1:1 AL65 + Xp 5-6', which inoculates strains AL65 and Xp 5-6 at a 1:1 ratio, the 8 ml of 10^8 cfu/ml of bacteria would be comprised of 4 ml of 10^8 cfu/ml AL65 and 4 ml of 10^8 cfu/ml Xp 5-6 and diluted in the same manner as described above; likewise, to inoculate strains AL65 and Xp 5-6 at a 3:1 ratio, the 8 ml of 10^8 cfu/ml of bacteria would be comprised of 6 ml of 10^8 cfu/ml AL65 and 2 ml of 10^8 cfu/ml Xp 5-6 (Table 1). To negate any possible interbacterial interactions prior to dip inoculation, the 10^8 cfu/ml of bacterial suspensions were kept separate in conical tubes and only introduced to

the dilutant/Silwet L-77 solutions, which were contained within 1000 ml graduated media bottles for easy mixture in the greenhouse by vigorous shaking, at the time of inoculation.

Table 3-1. Preparation of Inoculum. For preparing an inoculum (800 ml) of 10^6 cfu/ml of bacteria in 0.01 M $MgSO_4$ buffer with 0.0025% Silwet L-77 (v/v) containing one strain (monostrain treatment), 8 ml of 10^8 cfu/ml bacteria (Pathogen 1 OR Pathogen 2, Pathogen 1 is seen here) suspended in 0.01 M $MgSO_4$ can be mixed into 792 ml of sterile 0.01 M $MgSO_4$ supplemented with 20 µl Silwet L-77 (0.00025%; v/v). Likewise, for multistrain treatments, prepare inoculum the same way, but substitute the 8 ml of 10^8 cfu/ml bacteria with a ratio of two bacteria each at 10^8 cfu/ml: for a 1:1 ratio of Pathogen 1:Pathogen 2, the 8 ml of bacteria will be made up of 4 ml of 10^8 cfu/ml Pathogen 1 and 4 ml of 10^8 cfu/ml bacteria Pathogen 2, and for a 3:1 ratio, the 8 ml of bacteria will be made up of 6 ml of 10^8 cfu/ml Pathogen 1 and 2 ml of 10^8 cfu/ml bacteria Pathogen 2.

Treatment	**Monostrain**	**Multistrain**	**Multistrain**
Ratio of Pathogen 1: Pathogen 2	1	1:1	3:1
Pathogen 1 (10^8 cfu/ml in 0.01 M $MgSO_4$ buffer)	8 ml	4 ml	6 ml
Pathogen 2 (10^8 cfu/ml in 0.01 M $MgSO_4$ buffer)	n/a	4ml	2 ml
0.01 M $MgSO_4$	792 ml	792 ml	792 ml
Silwet L-77 (0.0025%; v/v)	20 µl	20 µl	20 µl
Total Inoculum (10^6 cfu/ml)	800 ml	800 ml	800 ml

3. Following preparation of a treatment's inoculum in the greenhouse, pour the inoculum into a 1000 ml beaker before proceeding to individually dip the phyllosphere portion of each

plant within the treatment by inverting the plant into the inoculum for 30 seconds. After treating, remove the plant from the treatment inoculum and allow the excess to drop above the inoculum beaker before setting aside to dry (Figure 3-3). Repeat for each treatment and a control treatment (using 800 ml 0.01 M $MgSO_4$ amended with 20 µl of Silwet L-77) ensuring no cross-contamination occurs between treatments.

Note: Plants used in this experiment were 4-5 week old Florida 47 R tomato plants at the time of inoculation.

Figure 3-3. Infecting Plants by Dip Inoculation Inoculation of a 4-week old tomato plant by dipping into a solution of 10^6 cfu/ml bacteria for 30 seconds

4. Continue to 'Microbe Collection from Plant Tissue' on 1, 4, 8, and 12 days post inoculation (DPI).

C. Microbe Collection from Plant Tissue

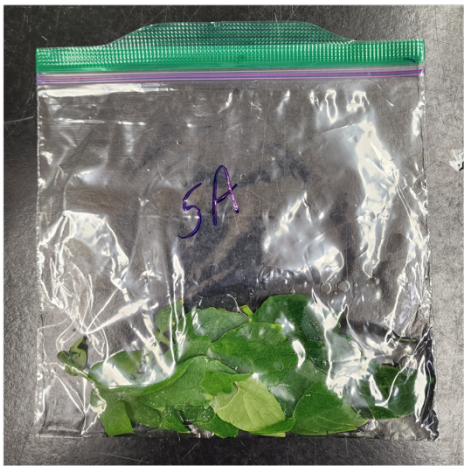
1. For each treatment, retrieve 9-12 leaflets and place inside of a sandwich bag. Tare the weight of an empty sandwich bag and record the final weight of the leaf tissue sampled. Depending on the amount of leaf tissue, add 15-20 ml of 0.01 M $MgSO_4$ buffer using a sterile pipette while ensuring even liquid coverage over the leaves. Remove all the air from the sandwich bag before sealing tightly (Figure 3-4.A).

   Note: One best practice to ensure even coverage of liquid among the leaves and uniform sonication would be to organize the leaf tissue towards bottom of the sandwich bag.

2. Following the addition of water to the sonicator bath, place the bottom of the sandwich bag with the leaf tissue and buffer into the water without submerging the zipper portion of the bag, this can be done by either hanging the top portion over the sonicator basket/wall or by suspending the bags into the water (Figure 3-4.B).

   Note: The pathogen used to develop this protocol is a foliar pathogen which maintains colonization on the epiphytic surface of leaves before entering the plant through stomata to colonize the apoplast – to recover bacteria from leaves infected by this pathogen, sonication is used to dislodge those bacteria from both the apoplast and epiphytic surface. In the event that sonication is unfit for a particular experiment, other methods can be implemented for microbial collection.

A.



B.

Figure 3-4. Microbe Collection from Plant Tissue: Addition of Buffer to Plant Tissue and Placement of Samples in Sonicator. A. Plant tissue sample amended with 0.01 M $MgSO_4$ Buffer that is evenly distributed among the sample. B. Placement of multiple buffer-amended samples within the sonicator bath.

3. Once the bagged samples are placed in the sonicator bath, weigh the bottom of the bags down with a lightweight object to ensure the sample is immersed in the water throughout sonication. Turn the sonicator on and run for 15 minutes while maintaining a bath temperature < 25°C by placing ice in the water before starting and/or over time (Figure 3-5).

Caution: If the bottom of the bags are sitting in the sonicator bath in such a way that they appear to have an unequal amount of buffer dispersed among the leaves, it may be beneficial to reorient the bags by flipping them over halfway through sonication before continuing.

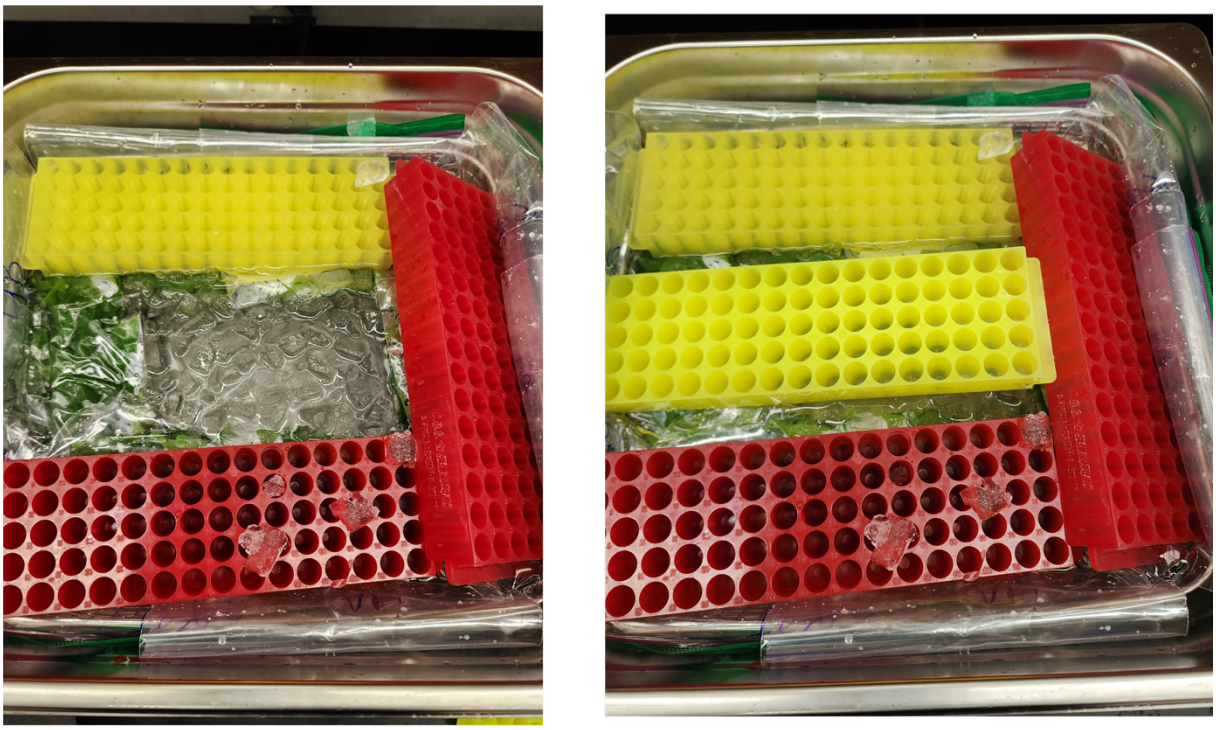


Figure 3-5. Microbe Collection from Plant Tissue: Addition of Ice into the Sonicator and Addition of Lightweight Objects to Ensure Submergence of Samples Addition of ice into the sonicator to help reduce degradation of leaf tissue following inevitable temperature increase during sonication. Lightweight objects are placed on top of the samples helping to submerge them within the sonicator bath water and to ensure proper sonication of samples.

4. Removing the bagged samples from the sonicator bath and using a sterile serological pipette, transfer the buffer from each bag into a labelled conical tube while squeezing the

leaves to recover as much buffer as possible (Figure 3-6). Balance the liquid level across all conical tubes before centrifuging at 785g for 30 minutes.

Note: Depending on the amount of leaf material, the inability to recover ~5 ml of buffer from the sonicated leaves is normal.

See "Troubleshooting: Problem 1, Solution 1"

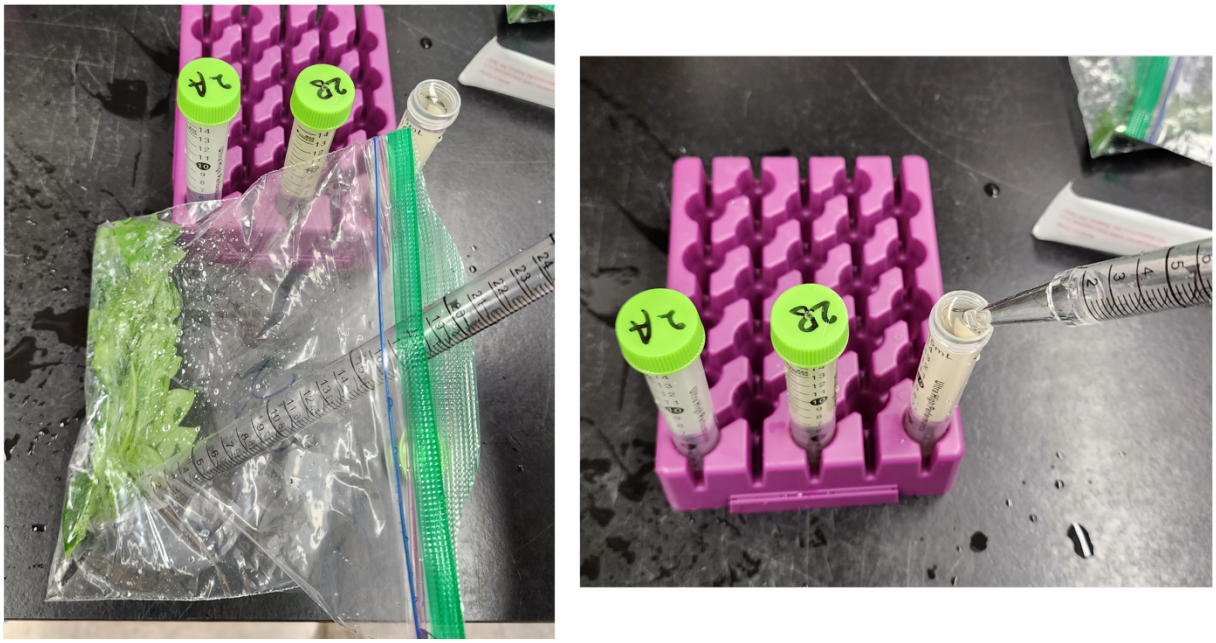


Figure 3-6. Microbe Collection from Plant Tissue: Removal of Sonicated Buffer from Plant Tissue Sample Prior to Centrifugation. Sonicated buffer being transferred using a serological pipette from the sonicated plant tissue to a conical tube prior to centrifugation of the sample to separate the microbial pellet from the buffer.

5. Following centrifugation, discard the supernatant and resuspend the pellet in 500-1,000 µl of filter-sterilized buffer depending on the estimated bacterial load; tare the weight of an empty conical tube and record the weight of the suspension. Vortex the suspension and use

a 70 μm cell strainer to strain the suspension into a sterile, labelled microcentrifuge tube. In the case of not having cell strainers, allow large debris to settle for 1 minute before removing just enough gravity-separated supernatant to not disturb the debris.

See "Troubleshooting: Problem 1, Solution 2"

6. Place all the samples on ice to prepare for input into the flow cytometer.

D. Flow Cytometry and Enumeration of Bacteria

1. Run each sample through a flow cytometer (CytoFLEX LX from Beckman Coulter was used within this experiment) at a slow, medium, or fast speed depending on the density of microbes within the sample to ensure the abort rate stays low, preferably below ~10-15%. Run each sample until at least 15,000 events have been recorded within a gate including all bacteria of interest (such as size in this case). When switching samples, allow for the machine to process liquid for 30 seconds before beginning to record the sample to reduce cross-contamination of sample data collected.

   See "Troubleshooting: Problem 2"

   Note: In the event that the microbe density was extremely low within a particular sample to where recording 15,000 events would take a very long time, the sample was processed by the flow cytometer for a total of 5 minutes.

2. Calculate population of bacteria (bacteria/g of leaf tissue) using the weight of the leaf tissue prior to addition of buffer or sonication (g), the weight of the suspension of the pellet in filter-sterilized buffer following centrifugation and removal of the supernatant (g directly proportional to ml), the amount of liquid processed by the flow cytometer (ml), and the number of events counted by the flow cytometer within the gates set up prior to

the experiment.

See "Data Analysis: Equation 3-1".

Data analysis

With analysis of flow cytometry data collected from monostrain treatments being more straightforward and essentially within the analysis of multistrain treatments (which are the main focus of the development of this protocol), an overview of those treatments coinoculated with different ratios of two strains is presented below.

To begin analyzing the flow cytometry data, we created gates based on the size (forward scatter area - FSC-A), granularity (side scatter area - SSC-A), and fluorescence intensity (FITC-A or PE-A) of fluorescently labelled bacterial strains. Using those gates, firstly, the bacteria used in this experiment can be separated from the majority of resident phyllosphere microbiota by a FSC-A/SSC-A dot plot in which a '*Xanthomonas perforans*' gate was created based on bacterial size and granularity of pure cultures (Figure 3-7). Those events within '*Xanthomonas perforans*', are then separated based on their placement within a quadrant of FITC-A/PE-A (fluorescent channel area where sGFP2 was detected on fluorescent channel FITC and mScarlet-I was detected on fluorescent channel PE) where events within the upper left quadrant represent those positive for only mScarlet-I, the lower right quadrant only sGFP2, the upper right quadrant both mScarlet-I and sGFP2, and the lower left quadrant neither fluorescent protein (Figure 3-8). Counts of bacteria within the lower right quadrant positive for only sGFP2 were used in calculation of the amount of AL65/g of leaf tissue, and counts of bacteria within the upper left quadrant positive for only mScarlet-I were used in calculation of the amount of Xp 5-6/g of leaf tissue.

Equation 3-1.

$$\frac{\frac{count\ of\ bacteria}{liquid\ processed\ through\ flow\ cytometer\ (ml)} * liquid\ supplemented\ to\ pellet\ (ml)}{leaf\ tissue\ (g)} = bacteria/gram\ of\ leaf\ tissue$$

After proper gating of the bacteria and subsequent retrieval of the number of cells, calculating populations of bacteria (bacteria/g of leaf tissue) using flow cytometry was achieved in this experiment by first calculating the amount of cells/ml of liquid processed by the flow cytometer, multiplied by the amount of liquid supplemented to the pellet following sonication (ml) to get the total amount of cells that existed within the sonicated buffer, then lastly dividing the total amount of cells by the grams of leaf tissue sonicated (Equation 3-1).

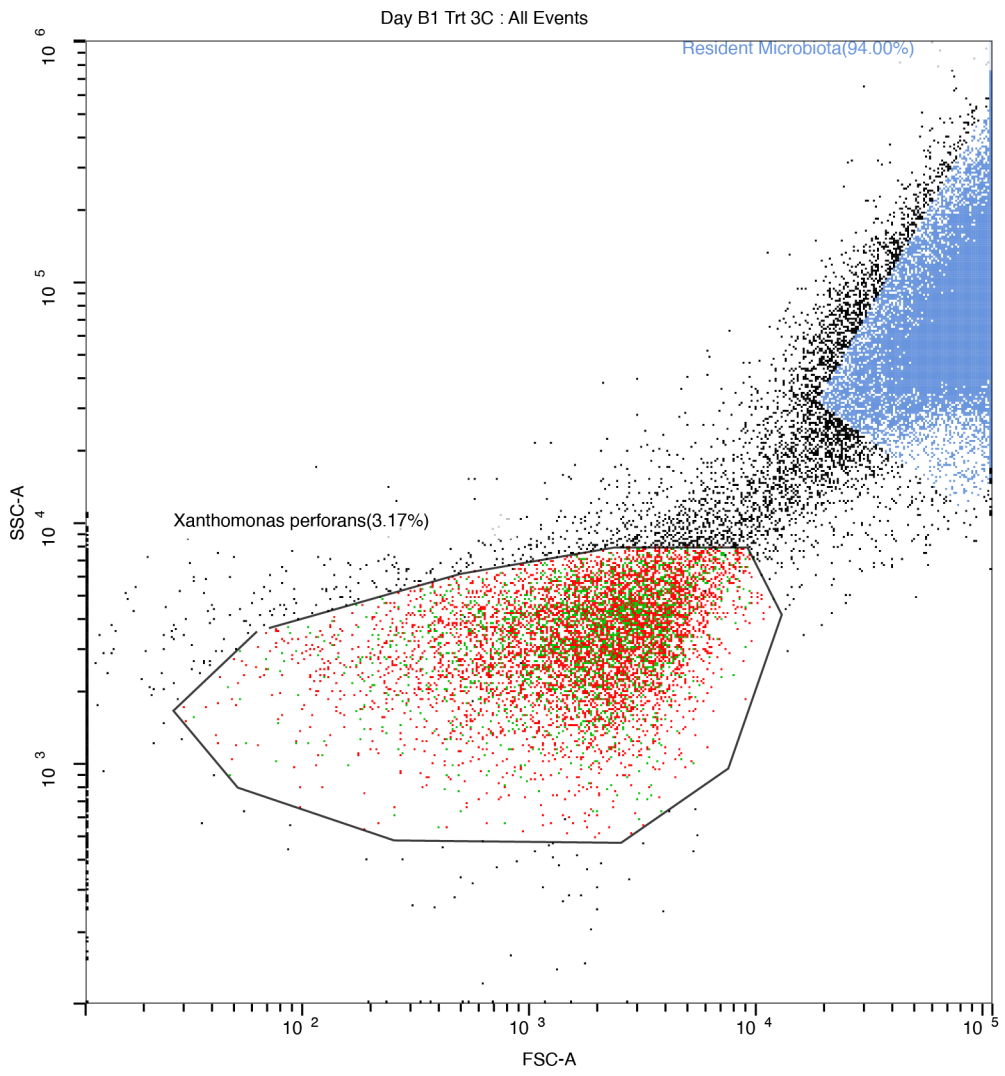


Figure 3-7. First Gate for Bacteria Processed by Flow Cytometry. *X. perforans* are first gated based on size (FSC-A) and granularity (SSC-A) where a small range of bacteria fit to that of pure cultures is designated as the '*Xanthomonas peforans*' inoculated. A 'Resident Microbiota' gate was also created, but was not used in the analysis.

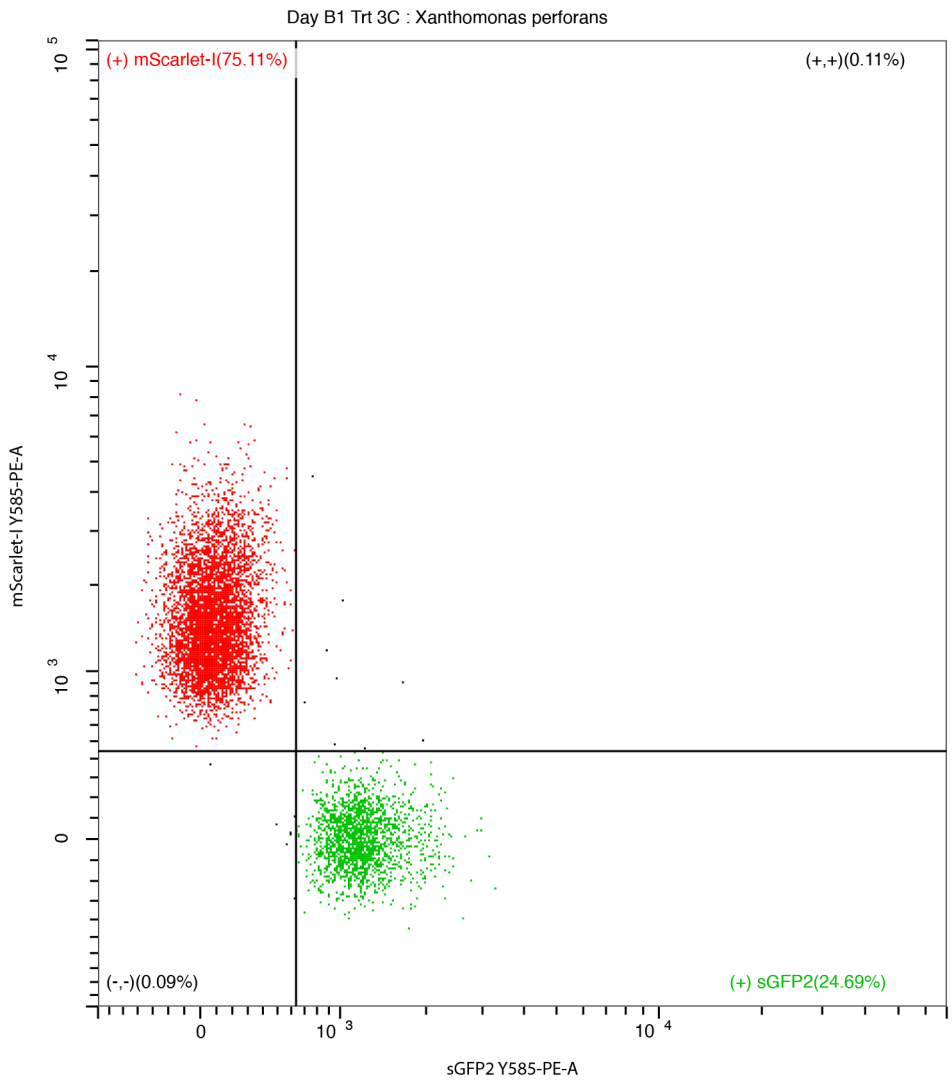


Figure 3-8. Second for Bacteria Processed by Flow Cytometry. After gating by FSC-A/SSC-A, cells are then gated based on intensity and presence or absence in both, neither, or only one fluorescent channel. Events within the upper left quadrant represent those positive for only mScarlet-I, the lower right quadrant only sGFP2, the upper right quadrant both mScarlet-I and sGFP2, and the lower left quadrant neither fluorescent protein. These upper left and lower right gates were used directly to get counts for the two *X. perforans* strains used in this experiment.

General notes and troubleshooting

General notes

1. To distinguish co-inoculated bacteria with flow cytometry, a few criteria must be met: firstly, the bacteria must not have a high autofluorescence already at the channel of the fluorescent markers; secondly, the fluorescent markers must be distinguishable from each other when ran through the flow cytometer together; and thirdly, the fluorescent markers should not be able to be readily lost from the cell during the experiment such in the situation that they are plasmid-bound, it is best that they are chromosomally integrated.

2. Not only is there possible bias the plate when using culture-based methods with multiple microbes, one other reason to use culture-independent methods is the fact that culture-dependent methods can take a lot of time to conduct and can require a lot of resources. Depending on the size of the experiment the correct amount of plates must be poured and amended with different antibiotics if that is the distinguishing marker between the bacteria; then following acquisition of the bacteria from the plants using any preferred method, the proper dilution must be prepared (which, mind you, if the dilution is uncertain the proper dilution may not be plated, unless multiple plates are used to plate multiple dilutions (costing more money), and thus, an entire data point may be missed) and then days must be set aside for growth of the bacteria before counting each plate. With culture-independent methods, if something like this protocol's "Troubleshooting: Problem 2" is encountered and the density of microbes is too high, the fix can be as simple as diluting the sample with more buffer – with other reasons tying into a lower cost and time-commitment, culture-independent methods can start to look more and more appealing if optimized for an experiment.

Troubleshooting

Problem 1: Plant material is abundant within the buffer following sonication

Possible cause: Various factors such as cleanliness of the plant sample or decreased physical integrity of the plant tissue could result in more debris being present in the buffer following sonication.

Solution 1: Large debris can be separated from the suspension of sonicated bacteria within the buffer by allowing the debris to settle for a short period of time (30 seconds to 2 minutes depending on the size of the debris particles) before transferring the density-separated supernatant to a clean tube.

Solution 2: Debris can also be filtered out using cell strainers post-centrifugation.

Problem 2: The abort rate on the flow cytometer is consistently too high

Possible cause: There may be debris within the sample (see "Troubleshooting: Problem 1" and subsequent solutions above) or the density of microbes within the sample could be too high, thus the microbes could be sticking together or could be passing by the flow cytometer's laser in a large crowd conducive to a higher abort rate.

Solution 1: See "Troubleshooting: Problem 1, Solutions 1 and 2".

Solution 2: The density of microbes can be diluted using filter-sterilized buffer to help decrease the abort rate. As the population of bacteria *in planta* increases, the amount of filter-sterilized buffer added to the pellet of microbes to be analyzed with flow cytometry will need to be optimized. Additionally, the flow rate for these high-density samples can be reduced to hopefully reduce the abort rate.

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Competing interests

The authors declare that they have no competing interests.

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4. CHAPTER FOUR

Coinfection of closely related strains from two intraspecific lineages of *Xanthomonas perforans* on tomato and its influence on disease dynamics

Abstract

Coinfection of pathogens is common in agricultural settings, but effects of coinfection on pathogen and disease dynamics are not well documented especially in systems of intraspecies coinfection. In this study, we looked at the effects of coinfection of two closely related strains from different lineages of the same species: *Xanthomonas perforans*, on both the assembly and succession of those strains within the plant and on disease severity outcomes. We found that population dynamics of coinfecting strains differed over time from initial concentrations and that coinfection could lead to higher disease severity than monoinfection, depending on monoinfecting strain.

Introduction

The response of a host plant to pathogen infection is variable and largely relies on virulence factors employed by the pathogen that suppress basal host defense mechanisms and allow for colonization of the plant (Gürlebeck, Thieme, and Bonas 2006). With the virulence factors variable across not only species to species (interspecifically) but also strain to strain within the same species (intraspecifically), disease dynamics of a pathogen and its host can be unpredictable at times and can pose issues when studying and developing management strategies. To complicate things more, from the beginning, plant pathological studies have compounded on themselves and evolved into an array of complicated and specialized research

areas. With general studies historically focusing on systems of one host to one pathogen, recent research has uncovered that, rather than existing as simple interactions, disease often combines multiple pathogenic species on the same plant where these coinfections have the ability to alter disease dynamics, sometimes leading to higher disease severity (Abdullah et al. 2017; Susi et al. 2015; Sadhukhan, Jacques, and Potnis 2023). As compared to infection with a single pathogen (monoinfection), coinfections can also alter pathogen dynamics within the host where not only does the host play a role in assemblage of the pathogen population (based on those virulence factors mentioned), but interactions of the coinfecting pathogens, either positive or negative, can play a large part as well (Abdullah et al. 2017; McLeish et al. 2019; Mehl and Cotty 2013). Studies have shown that assemblage of coinfecting pathogens is variable based on these pathogen-pathogen interactions but may also rely on priority effects where early-arrival of pathogens can alter host susceptibility to subsequent infection of additional strains (Jokinen et al. 2023; Halliday et al. 2020; Sadhukhan, Jacques, and Potnis 2023; Kinnula, Mappes, and Sundberg 2017; Bellah, Seiler, and Croll 2023b). Studies have also shown that simultaneous infections can be significantly more damaging to hosts than sequential infections where priority effects were evident (Marchetto and Power 2018). Within a coinfection, depending on the pathosystem where host resistance and virulence and competitive interactions of coinfecting strains vary, disease and pathogen assemblage outcomes remain an understudied topic in many systems.

With respect to a pathogen with a great amount of intraspecific genetic diversity, in the pathosystem of *Xanthomonas perforans* causing bacterial leaf spot (BLS) on tomato (E. A. Newberry et al. 2019; Adhikari et al. 2019; Timilsina et al. 2020), there has been an ongoing issue with efforts for breeding for resistance because of the difficulty in finding a single-gene

target due to this diversity (Abrahamian, Klein-Gordon, et al. 2021). This species is a worldwide pathogen not only infecting tomatoes but peppers as well, and it is considered endemic to the Southeastern U.S. where this lack of a solid management strategy poses a major risk to farmers crop security. In 2019, the intraspecific diversity within *X. perforans* was profiled by describing six different 'sequence clusters'. A sequence cluster (SC) is simply a metric of grouping strains within the species that are more closely related to each other than to strains of other SCs using core single nucleotide polymorphism (SNP) alignment of available genomes of strains belonging to this species followed by Baysian hierarchical clustering into different lineages (E. A. Newberry et al. 2019). As described in Chapter 2, we conducted a study employing high resolution metagenomic approach on tomato leaf samples collected from the fields in the southeastern US (from the states of Alabama, Georgia, North and South Carolina) over the course of growing seasons in 2020, 2021 and 2022, and obtained strain-resolved pathogen profiles. This study identified which lineages (referred to as sequence clusters, SCs) were present in the fields at a given time. Certain SCs maintained similar relative abundances even when spatiotemporally variable. Out of the eight sequence clusters (updated from the previous 6) described and profiled, three in particular held relatively high abundances (SCs 3, 4, and 6) while the remaining five SCs were often found at low relative abundance as auxiliary SCs to those dominant in the field (SCs 1, 2, 5, 7, and 8) (Bhandari et al. unpublished data). Following the observation of multiple sequence clusters of *Xanthomonas perforans* co-occurring in the same field but at different relative abundances both spatially and temporally, and the finding that some sequence clusters tend to maintain similar dominant or auxiliary relative abundances regardless of spatiotemporal variations, this study aims to elucidate the interactions between the host plant

and pathogenic SCs and possible strain:strain (SC:SC) interactions that could be present *in planta*.

In this study we tried to address the question of how intraspecific diversity of *X. perforans* is assembled following simultaneous inoculation *in planta* to gain a deep look at coinfection of two closely related strains from different SCs: SC6 and SC2, of which one was often found at relatively high abundance (SC6) and the other was often found at a relatively low abundance in farm fields (Bhandari et al. unpublished data, Chapter 2). We hypothesize that the host plant and its interaction with virulence factors of particular SCs plays a role in how abundance is assembled, and that strain interactions between the SCs, positive or negative, may play a role as well. Additionally, with the conducted experiments, we addressed the question as to whether or not coinfection could influence disease severity and hypothesized that coinfection would increase disease severity as compared to a monoinfection.

Materials and Methods

Plant material and growth conditions for greenhouse *in planta* experiments

For all *in planta* experiments in this study, hybrid FL47R tomato plants were used. Seeds were planted in the greenhouse and grown until the two-week-old seedlings were transplanted into 4" plastic pots with potting mix and Osmocote® fertilizer. The plants remained under greenhouse conditions at 28°-30°C throughout the experiment and were inoculated at 4-5 weeks old.

Transformation of bacterial strains with fluorescent labels and growth parameters

Two bacterial strains were used in this experiment: AL65 and Xp 5-6 belonging to SC6 and SC2, respectively. These strains were transformed to express two different fluorescent proteins (mScarlet-I for SC2 [chloramphicol and gentamycin resistant] and sGFP2 for SC6 [chloramphenicol and kanamycin resistant]) using Tn7 transposon delivery plasmids by electroporation of extracted plasmids into the SCs (Schlechter et al. 2018). Fluorescently labeled bacterial strains were streaked from glycerol stocks onto nutrient agar (NA, Difco; supplemented with 15 µg/ml gentamycin for growing SC6 and 50 µg/ml kanamycin for growing SC2) and incubated at 28°C for 24-48 hours.

Dip inoculation of mono- and coinfection treatments *in planta* with fluorescently marked bacteria under greenhouse conditions

Five treatments and a control treatment were used in this experiment: with the two mono-strain treatments containing just AL65::MRE-Tn7-152 (belonging to and herein referred to as SC6) or Xp 5-6::MRE-Tn7-145 (belonging and herein referred to as SC2) were 'SC6' and 'SC2' respectively, and three multi-strain treatments coinoculating SC6 and SC2 at ratios of 1:1, 3:1, and 1:3, were '1:1 SC6 + SC2', '3:1 SC6 + SC2', and '1:3 SC6 + SC2', respectively. To begin preparing inoculum bacteria for each of these treatments, bacterial cultures of both *Xanthomonas perforans* SC6 and SC2 were grown overnight and each suspended in $MgSO_4$ buffer at a concentration of 10^8 cfu/ml (OD_{600} = 0.3). To create inoculum for the monostrain treatments 'SC6' and 'SC2', 8 ml of 10^8 cfu/ml of SC6 and SC2 respectively, were diluted 100x in 0.01 M $MgSO_4$ buffer supplemented with 20 µl (0.0025% = vol/vol) Silwet L-77 to form 800 ml of a final inoculum of 10^6 cfu/ml. For the coinfection treatments, inoculum preparation was the same

but with the 8 ml of 10^8 cfu/ml bacteria to be diluted being comprised of both SC6 and SC2 at different ratios: '1:1 SC6 + SC2' contained 4 ml of 10^8 cfu/ml SC6 and 4 ml of 10^8 cfu/ml SC2, '3:1 SC6 + SC2' contained 6 ml of 10^8 cfu/ml SC6 and 2 ml of 10^8 cfu/ml SC2, and '1:3 SC6 + SC2' contained 2 ml of 10^8 cfu/ml SC6 and 6 ml of 10^8 cfu/ml SC2. Bacterial suspensions constituting the 8 ml of 10^8 cfu/ml bacteria for each treatment were contained in conical tubes and were not diluted to form the final inoculum until the exact time of inoculation (the $MgSO_4$ dilutant/Silwet L-77 solutions were contained within 1000 ml graduated media bottles for easy mixture by vigorous shaking once the 8 ml of 10^8 cfu/ml bacteria was added), and, importantly, for the coinfection treatments, the two strains were kept in separate conical tubes and only mixed at the time of dilution to negate any possible interstrain interactions. Plants from each treatment were dip inoculated by inverting the 4-5 week old plants for 30 seconds into a beaker containing the treatments respective final inoculum of 10^6 cfu/ml bacteria. Control plants were dip inoculated with 800 ml sterile $MgSO_4$ amended with 20 µl (0.0025% = vol/vol) Silwet L-77. This experiment was repeated three times, and each time leaf samples were collected from each treatment on 1, 4, 8, and 12 days post inoculation (DPI) and disease severity of the entire phyllosphere portion inoculated was estimated by two individuals on 12 DPI using a scale of 0-100% with 0% disease severity meaning no disease and 100% meaning complete death of the plant.

Microbe collection from plant tissue and processing by a flow cytometer

For each treatment, 9-12 leaflets were collected and placed inside of a sandwich bag. After taring the weight of an empty sandwich bag, the final weight of each treatment's leaf tissue was recorded. Depending on the amount of leaf tissue collected, 15-20 ml of 0.01 M $MgSO_4$ buffer (essentially

enough to cover the plant tissue) was added to the bagged leaf sample using a sterile serological pipette before the bag was void of all air and sealed tightly. Following addition of water to a sonicator, the bottoms of the bagged samples where the leaf tissue resided were placed into the sonicator water and the zipper portions of each bag were hung over the basket/sonicator wall so as to not make contact with the water. Once all of the bags were placed in the sonicator bath, they were slightly weighed down using a lightweight object to ensure immersion in the water throughout sonication before the sonicator was turned on and the samples were subjected to sonication for 15 minutes. Throughout sonication, the bath temperature was maintained to be below <25°C by placing ice in the water before starting and/or over time. Upon completion of sonication, the bagged plant samples were removed and the buffer from each bag was transferred into labelled conical tubes using sterile serological pipettes; these tubes containing the sonicated buffer were then centrifuged at 785g for 30 minutes. Following centrifugation, the supernatant was discarded, and each treatment's pellet was resuspended in 500-1,000 µl of filter-sterilized 0.01 M $MgSO_4$ buffer depending on the estimated bacterial load. This suspension was then weighed and strained into a labelled microcentrifuge tube using a 70 µm cell strainer; all strained samples were put on ice and processed by flow cytometry (Russell et al. unpublished data, Chapter 3). Following gating of SC6 and SC2 using their respective fluorescent labels and record of the number of events recorded for each strain, which is detailed in Russell et al. (unpublished data, Chapter 3), the populations SC6 and SC2 within each treatment (bacteria/g of leaf tissue) were calculated.

Bacteriocin assay to assess antagonism between SC6 and SC2

Following a similar approach as Hert (2007), a bacteriocin assay was performed to assess bacteriocin production by SC6 (antagonistic test strain) against SC2 (overlay strain) and by SC2

(antagonistic test strain) against SC6 (overlay strain) (Aaron Paul Hert 2007). Firstly, 10 µl of the antagonistic test strain was drop-spotted onto the middle of a nutrient agar glass plate and grown at 28°C overnight before the formed antagonistic test strain colony was killed by inverting the glass plates over 3 ml of chloroform until all of the chloroform had evaporated. Next, 3.5 ml of 0.3% water agar (50°C) inoculated with 200 µl of $5x10^7$ cfu/ml overlay strain was overlayed on top of the plate containing the killed antagonistic test strain and let grow at 28°C before being checked after 24 hours for a zone of inhibition.

Data analysis

A detailed protocol of the use of flow cytometry in this study to assess population of SC6 and SC2 was published as a BioProtocol (Russell et al. unpublished data, Chapter 3). Analysis of disease development using disease severity percentage and pathogen dynamics using SC population and area under growth progress curve (AUGPC) were conducted and statistical tests of linear mixed effects model and post-hoc test Dunn tests were used.

Results

When coinfected on tomato plants under greenhouse conditions, SC6 predominates over SC2 regardless of initial concentration

Across all four timepoints, the control treatment (MgSO4 buffer inoculated plants) maintained 1-2 log populations of SC6 and SC2. SC6 and SC2 monoinfection treatments also showed presence of SC2 and SC6, respectively, based on analysis by flow cytometry. These levels of non-inoculated bacteria within the monoinfection treatments were similar to that of control on 1 DPI,

yet increased by 12 DPI. Additionally, by 12 DPI, SC6 in the SC2 monoinfection treatment reached higher levels than the non-inoculated SC2 in the SC6 monoinfection treatment. Throughout the experiment, the populations of inoculated versus non-inoculated bacteria in the treatments remained significantly different. For all coinfection treatments, SC6 persisted at a significantly higher population (Log bacteria/g of leaf tissue) than SC2 with all three treatments regardless of ratio, yet populations within the treatment inoculated at a 1:3 ratio of SC6 to SC2 were never found to be significantly different (Figure 4-1.A). On 1 DPI, no significant differences were found between the population of SC6 and SC2 regardless of ratio and population dynamics were similar to ratios inoculated, yet at a lower level. By 4 DPI, when inoculated at an unequal ratio of 3:1 a significant difference was seen between the two SCs where the population of SC6 was higher than that of SC2.These two populations remained significantly different throughout the experiment until 12 DPI. When inoculated at an equal 1:1 ratio of SC6 to SC2, by 8 and 12 DPI, SC6 is significantly higher than SC2. Area under the growth progress curves (AUGPC) were compared between SCs, and in both monoinfection treatments that showed the other non-inoculated SC, AUGPC values were significantly different. Across the coinoculation treatments, those inoculated at ratios of 1:1 and 3:1 showed significant differences between the SCs, yet no significance was found at a ratio of 1:3 (Figure 4-1.B). Additionally, across those coinoculation treatments, AUGPC values for SC2 showed more variation than that of SC6. more variation in treatments with ratios inoculated of 1:1 and 1:3 as compared to 3:1 SC6 to SC2.

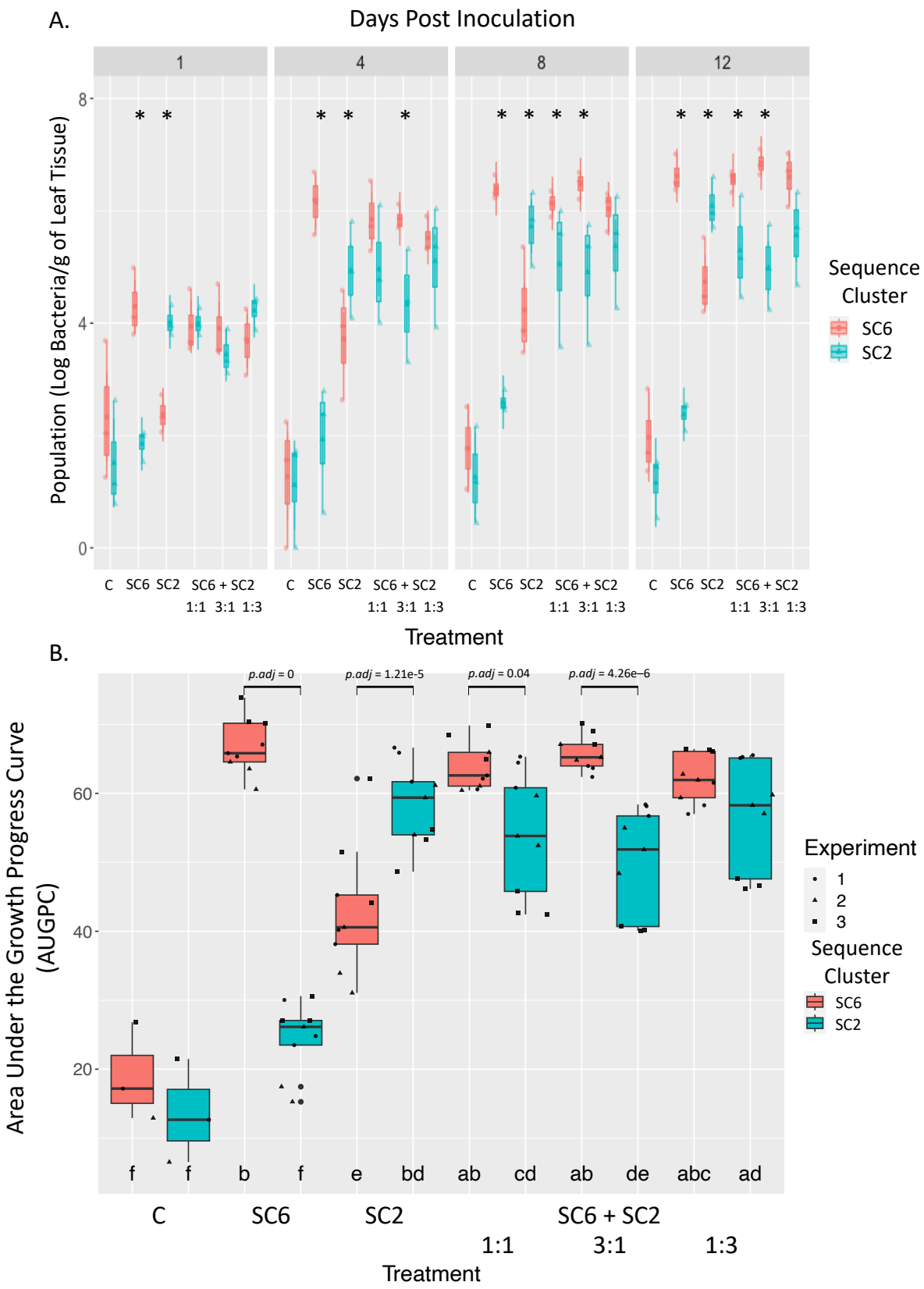
A.
Days Post Inoculation
1
4
8
12
Population (Log Bacteria/g of Leaf Tissue)
8
4
0
C SC6 SC2 SC6 + SC2
1:1 3:1 1:3
Treatment
Sequence Cluster
SC6
SC2
B.
p.adj = 0
p.adj = 1.21e-5
p.adj = 0.04
p.adj = 4.26e−6
Area Under the Growth Progress Curve (AUGPC)
60
40
20
f f b f e bd ab cd ab de abc ad
C SC6 SC2 SC6 + SC2
1:1 3:1 1:3
Treatment
Experiment
1
2
3
Sequence Cluster
SC6
SC2

Figure 4-1. Populations of strains from different SCs (Log bacteria/g of leaf tissue) when dip inoculated in planta at different ratios over four sampling timepoints when grown under greenhouse conditions. Four to five week-old tomato plants (cv. FL47R) were inoculated with ~1x10^6 cfu/ml of either SC6 or SC2 or different ratios (1:1, 1:3, and 3:1 SC6:SC2) of SC6 and SC2 under greenhouse conditions and their populations were sampled at 1, 4, 8, and 12 days post inoculation (DPI) using flow cytometry. The experiment was repeated three times and each experiment was considered a batch factor in linear mixed modeling (A.) Bacterial population across four timepoints over 12 days plotted for both SC6 and SC2 from three different experimental batches. Around 1-2 log bacteria were showing on average for the control treatment across all four timepoints, and within the SC6 and SC2 monoinfection treatments, SC2 and SC6 were present, respectively, though the non-inoculated bacterial populations were significantly different from the inoculated ones throughout the experiment (p<0.05 for all, linear mixed model). On 4 DPI, SC6 was significantly higher than SC2 within the treatment of the two inoculated at a 3:1 ratio (p<0.0005). By 8 DPI, coinfection treatments with inoculum ratios of 1:1 and 3:1 had significantly different populations of SC6 and SC2 (p=0.04 and p<0.0005, respectively). These populations were found to be significantly different on 12 DPI as well (p=0.006 and p<0.0005, respectively). (B.) Area under the growth progress curve (AUGPC) raw values from experimental batches plotted with grouping letters according to significant difference (p<0.05 in a linear mixed model). Populations showing as SC6 and SC2 were not significantly different within the control treatment. For monoinfection treatments SC6 and SC2 which both showed presence of the opposite bacteria as well, AUGPC was significantly different for both (p<0.0005 for both). For treatments inoculated at a 1:1 and at a 3:1 ratio of SC6 to SC2,

AUGPC was significantly different when comparing that of SC6 to that of SC2 (p=0.04 and p=4.26e-6, respectively).

Disease severity of mixed infection

Disease severity of FL47R tomato plants on day 12 of the monoinfection treatment containing SC2 was significantly lower than that of two coinfection treatments containing SC6 and SC2 inoculated at different ratios (Figure 4-2). While the monoinfection treatments and the coinfection treatments themselves were not significantly different from each other, the monoinfection treatment containing just SC2 was on average ~4% lower than that of the treatment containing just SC6, and disease severity values were significantly lower than those observed in coinfection treatments with ratios of SC6 and SC2 inoculated at a 3:1 and at a 1:3 ratio ($p.adj$<0.005 for both, Dunn Test using Bonferroni method).

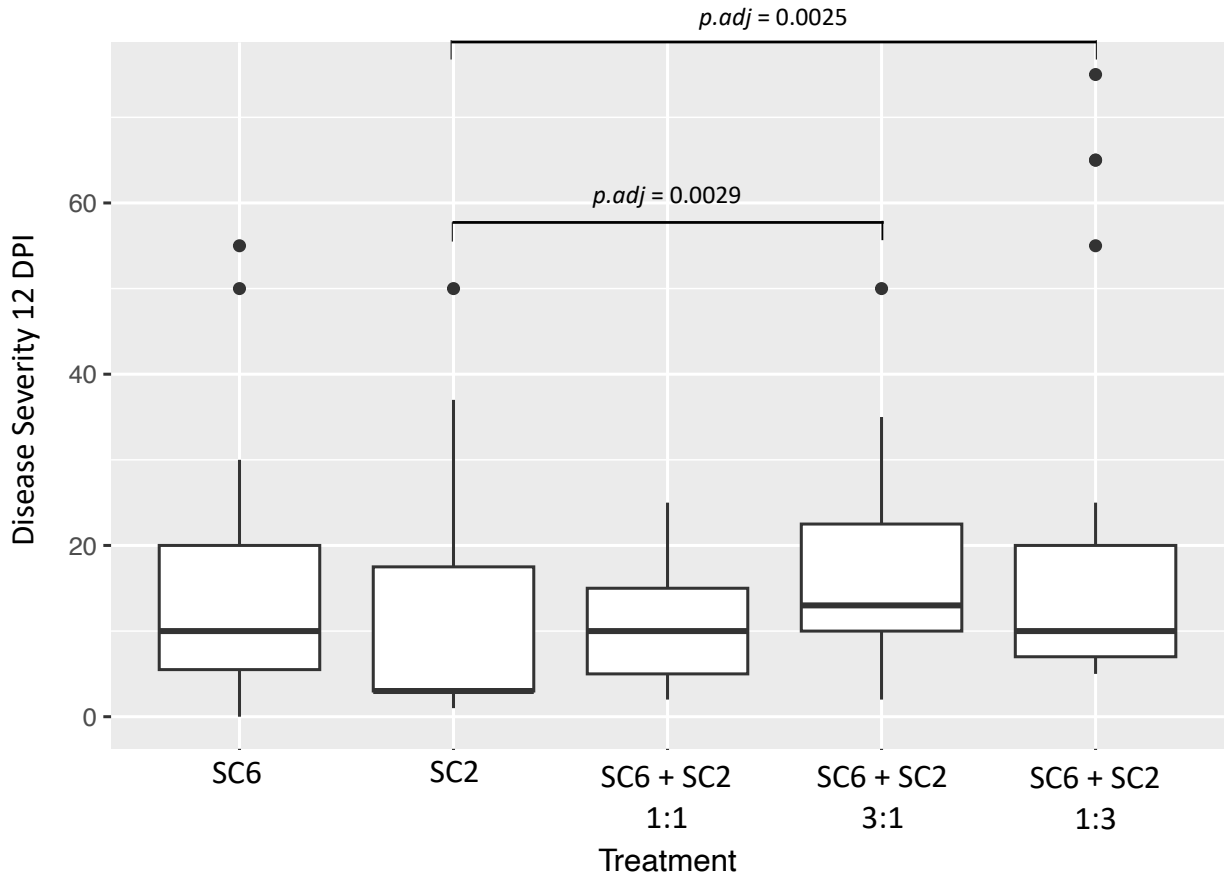

Figure 4-2. 12 DPI disease severity (on a scale of 0-100%) of FL47R tomato plants inoculated with different ratios of SCs. Four to five week-old tomato plants (cv. FL47R) were inoculated with ~1x10^6 cfu/ml of different ratios (1:1, 1:3, and 3:1 SC6:SC2) of SC6 and SC2 under greenhouse conditions and their disease severity was sampled on 12 DPI using a scale of 0-100% where 0% means no disease based on necrotic spot symptoms and 100% means complete death of the plant. Monoinfection treatments containing just SC6 or SC2 maintained average disease severity ratings of 14.89% for plants inoculated with SC6 and 10.59% for plants inoculated with SC2. Coinfection treatments with ratios of SC6 to SC2 of 1:1, 3:1, and 1:3 maintained disease severity ratings at or above 10% on average with the treatment inoculated with a ratio of 1:1 maintaining an average of 10.33%, 3:1 an average of 17.04%, and 1:3 maintaining the highest average disease severity ratings at 19.74%. Disease severity across

monoinfection nor across coinfection treatments was not significantly different, however, a significant difference between Day 12 disease severity of the monoinfection treatment with SC2 and the coinfection treatments with ratios inoculated of 3:1 and 1:3 were significantly different (*p.adj*=0.0029 and *p.adj*=0.0025, respectively, Dunn Test using Bonferroni method).

A bacteriocin assay to determine antagonism between SC6 and SC2 showed possible production of bacteriocins against SC2 by SC6

Using an overlay concentration of 200 µl of $5x10^7$ cfu/ml into 3.5 ml of 0.3% water agar, no clear zone of inhibition was observed surrounding either antagonistic test strain (Figure 4-3). Yet, a slight zone of inhibition may be seen around the SC6 colony overlayed with SC2 after 24 hours of incubation.

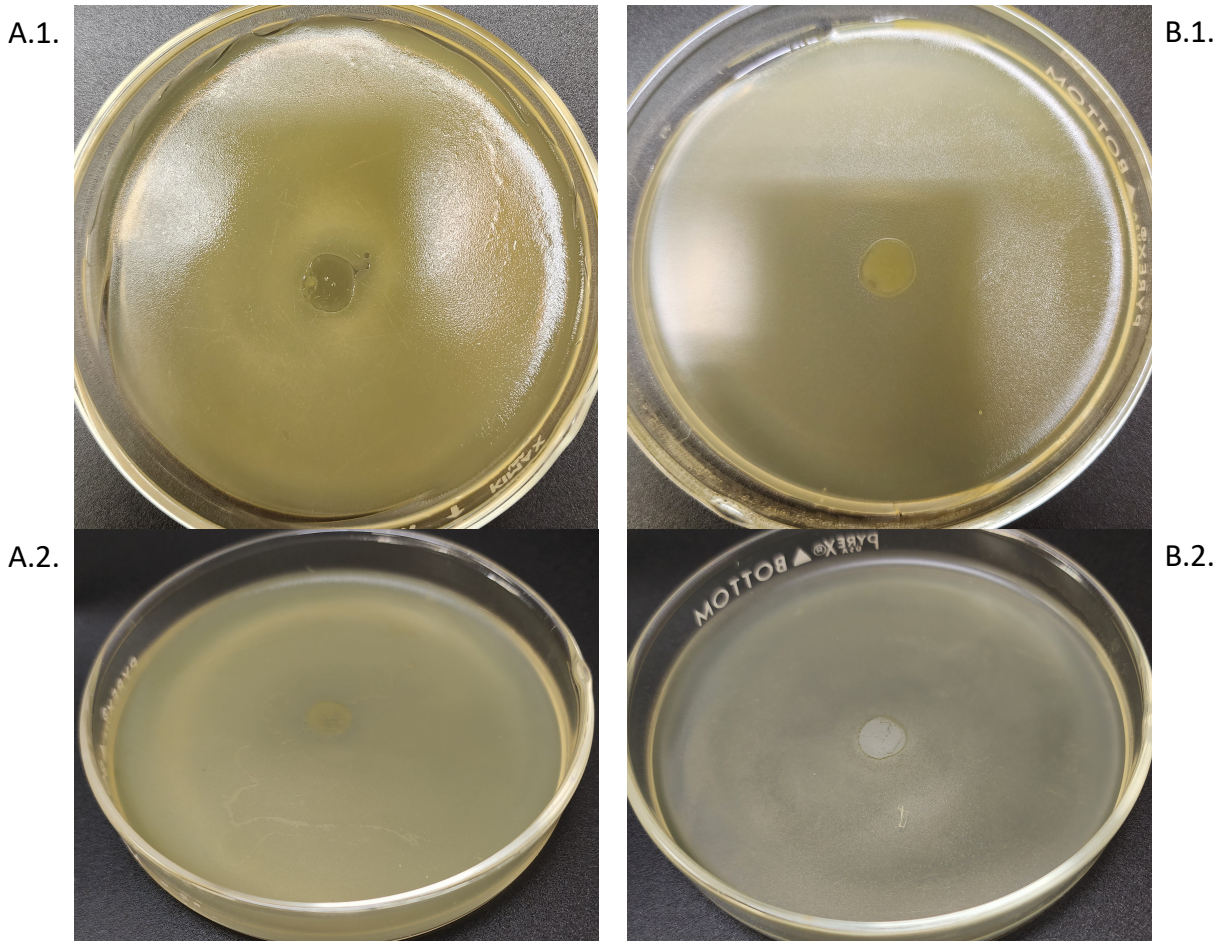

Figure 4-3. Bacteriocin assay conducted between SC6 and SC2. Bacteriocin assay performed by overlaying SC2 over a chloroform-killed colony of SC6 (Left) and by overlaying SC6 over a chloroform-killed colony of SC2 (Right) with two replicates for each (A/B.1. and A/B.2.). No zone of inhibition is prevalent with either SC6 against SC2 (A.1. and A.2.) or SC2 against SC6 (B.1. and B.2.), yet a small zone may be present with SC6 against SC2.

Discussion

This study marked an attempt to profile pathogen and disease dynamics within *X. perforans* whereby two closely related strains from different SCs of the species were coinoculated on tomato. Three *in planta* greenhouse experiments were conducted to address pathogen population assembly of SCs following inoculation at equal or unequal ratios of both SCs, and to determine whether or not disease severity of the host plant could be exacerbated when coinfected with the two SCs as opposed to a monoinfection of only one. Based on analysis with flow cytometry, we did find presence of both SC6 and SC2 within the control treatment plants at low but similar levels relative to each other and within the monoinfection treatments of just SC6 and SC2, yet at significantly lower population levels that those SCs actually inoculated (Figure 4-1.A). Prior to conduction of the three experimental runs analyzed here, optimization experiments using the same two fluorescently labeled SCs were conducted. Although *X. perforans* is known for its rapid movement and ability to spread through tomato production transplant houses and fields through wind (in the greenhouse, wind from fans), water, aerosols (>1 meter dispersal), or cultural practices (such as watering with the same hose and nozzle or brushing against the plants during routine work), the presence of SC6 and SC2 through flow cytometry analysis was likely not due to actual bacterial contamination within the greenhouse but rather during post-sampling processing (Abrahamian, Sharma, et al. 2021). Considering that levels of SC6 and SC2 within the greenhouse remained consistent within the control treatment across all four time points and were never significantly different from each other, firstly, it is possible that there are basal levels of plant autofluorescence within the gates set for *X. perforans* analysis (Doležel, Greilhuber, and Suda 2007; Donaldson 2020). Additionally, studies have reported carryover between samples used in flow cytometry which could explain not only the

discrepancy between the lower amount of SC2 found in the monoinfection SC6 treatment and the higher amount of SC6 found in the monoinfection treatment of SC2 which was sampled after that of SC6, but also specifically why the amount of SC6 in the SC2 treatment increases by 12 DPI (Van Nevel et al. 2013; Andersen et al. 2020). As to the slight increase over time in the SC2 found in the SC6 monoinfection treatment that would not be explained by carryover from the control treatment, autofluorescence within plants has been shown to change during pathogen infection which could mean changes in auto fluorescent signatures of the infected plants over time could be reflecting higher contaminant levels of SC6 and SC2 (Harding and Teakle 1988; Bellow et al. 2013; Cuzick, Urban, and Hammond-Kosack 2008; Bélanger et al. 2011). Additional studies could be done to address definite basal levels of fluorescent signatures and whether they are changing over time under infection using non-fluorescent *X. perforans* if this presence of SC6 and SC2 is due to autofluorescence, and an assessment of whether carryover is affecting samples could be conducted. Regardless of these issues, it is possible that samples have been contaminated by the buffer used to process the samples, and further testing could rule that out. While a repeat and adjustment of these experiments is necessary to reach solid conclusions regardless of whether or not the conclusion would change, this first full test run using flow cytometry to study *X. perforans* bacterial populations *in planta* under greenhouse conditions marks a valuable expansion of the way bacterial studies can be conducted.

Onto the results of the greenhouse experiments, we found that population values of SC6 and SC2 existing in a coinfection inoculated at different ratios on tomato plants resulted in an arrangement where SC6 maintained higher log bacteria/g of leaf tissue when inoculated at an equal ratio of 1:1 SC6 to SC2 and at an unequal ratio of 3:1 but not at a ratio of 1:3 (Figure 4-1.A). In addition to log population differences, AUGPC of SC6 was significantly higher than

SC2 when inoculated at ratios of 1:1 and 3:1, yet not significantly different when inoculated at 1:3 (Figure 4-1.B). Studies within pathosystems supporting a high level of within-host diversity with multiple intraspecific variants existing throughout infection having observed dominance of particular strains or genotypes over others when coinfected together which is similar to what is observed here, yet the underlying reason for such assemblages is not clear (J. A. Smith et al. 2010). A pathosystem is not only affected by interactions of two factors, the pathogen and the host, but also inter- and intraspecific pathogenic interactions and environmental parameters (Abrahamian, Klein-Gordon, et al. 2021; Potnis 2021). Considering that this general dynamic was observed in fields with SC2 persisting generally at a lower population than other SCs and SC6 generally at a higher population than others, this assemblage seems to be independent of environmental variation across the Southeast U.S. leaving possibly host x pathogen or pathogen x pathogen interactions to drive this assemblage (Bhandari et al. unpublished data). The dominance of SC6 over SC2 within these host x pathogen and pathogen x pathogen dynamics can have three plausible explanations *in planta*: (1) SC6 inhibits SC2 mediating competitive interactions; (2) SC6 has overall higher within-host multiplication capability than SC2; and (3) SC6 has higher transmissibility than SC2, thus allowing to maintain higher populations.

With SC6 having a higher population than SC2 when inoculated at a ratio of 1:1, it is possible that there is some negative competitive interaction between the two with SC6 prevailing over SC2 (Abdullah et al. 2017). A bacteriocin assay was performed and it does seem as though there may be bacteriocin production by SC6 against SC2, but the experiment could be repeated again with a lower amount of bacteria in the overlay to definitely observe an effect of antagonism using bacteriocins (Figure 4-3). Otherwise, the host may be mediating assemblage of SC6 and SC2 if the host is more susceptible (there is no host resistance in tomato, yet

susceptibility is variable) to virulence factors carried by the possibly more complex SC6. For context, *X. perforans* was predated by the closely related species *X. euvesicatoria*, and SC6 has been shown to have recombined largely with *X. euvesicatoria* related pathovars, *X. euvesicatoria*, and species outside of the *X. euvesicatoria* complex rather than primarily (and making up a smaller proportion of the core genome) *X. euvesicatoria* as in SC2 based off of a recent analysis by Newberry et al. (2019). Further, the strain representing SC6, AL65, was found to carry a plasmid often found in *X. cynarae* pv. gardneri with the type 3 secreted effectors XopAO and the TALE AvrHah1 with AvrHah1 being associated with increased virulence on tomato and pepper (E. A. Newberry et al. 2019) while the strain representing SC2, Xp 5-6, has been shown to lack an important effector for virulence in pepper (AvrBsT), effectively reducing its host range on peppers with strain specific virulence not documented but possibly lacking on tomato as well (Schwartz et al. 2015). Given these differences between the strains with SC6 possibly having an upper hand in terms of virulence factors than SC2, it is possible that it could have higher within-host multiplication capability than SC2 or it could have higher transmissibility, thus allowing it to maintain a higher population.

Disease severity results from this experiment show a twofold observation in which (1) SC2 alone is slightly less virulent compared to SC6 when inoculated individually, although not statistically significant, and (2) mixed infection with SC6 enhances disease severity levels in the treatments with an unequal ratio of initial inoculum (Figure 4-2). Interestingly, the treatment with the highest average disease severity on Day 12, i.e. with an initial inoculum ratio of 1:3 (SC6 to SC2), was the treatment without a significant difference in AUGPC of SC6 and SC2 (Figure 4-1.B and Figure 4-2). Studies have shown that coinfection can increase virulence of infection and influence disease outcome based on characteristics of the coinoculated strains and

their interactions (Kinnula, Mappes, and Sundberg 2017; Abdullah et al. 2017; Susi et al. 2015; Sadhukhan, Jacques, and Potnis 2023). Assuming that the extent of competitive forces was lower with initial inoculum ratio of 1:3 (SC6 to SC2), the mixed infection at this initial inoculum ratio leads to co-existence of both strains, rather than dominance of one over the other. This maintenance of heterogeneity within the pathogen population was observed to leading to the highest disease severity levels.

This work provides a baseline understanding of not only a new approach to conducting population studies *in planta* using flow cytometry, but also of high-resolution coinfection dynamics of two SCs within *Xanthomonas perforans*. Although more complex, the importance of studying coinfection dynamics at such a high resolution within pathosystems lies in the fact that disease dynamics may be altered following the introduction of more strains. With interesting results regarding how SC6 and SC2 assemble *in planta* and how their coinfection increases disease severity in some cases, this experiment opens the room to studies of other SC coinfections to hopefully create a better understanding of pathogen dynamics in farm fields.

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5. CHAPTER FIVE

Note: this chapter is a manuscript in progress that we plan to submit before the end of this year. My contributions are that helped to conduct the experiment, I did all of the analysis for and created Figure 5-1, I ran the relative abundance analysis using StrainEst, found the SNV sites in which minor alleles which were increasing in frequency from < 0.2 to > 0.8 from mid- to end-season and I also profiled which strain the alleles at the site matched to. Considering that this will be submitted before graduation, I am formatting the following chapter in the way of submission to the journal.

Rapid adaptation of a foliar pathogen, *Xanthomonas*, from gene to population level to pepper in presence of host resistance and ozone stress in a single growing season

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Abstract

Climatic fluctuations pose a growing threat of altered plant disease dynamics, with expanded host or geographical ranges. Disease resistance is the most effective and environmentally sound approach to manage diseases. However, we lack understanding of how climatic fluctuations may alter plant-pathogen interactions and what efficient ways would be to manage the disease. In this study, we assessed the efficacy of disease resistance in pepper against a leaf spot pathogen,

Xanthomonas perforans, under elevated ozone in open-top chamber field conditions. The study design also involved use of two pathogen genotypes, mimicking co-occurrence of different pathogen genotypes in pepper fields. We observed a higher disease severity on the resistant cultivar under elevated ozone. Such compromised disease resistance may be due to alteration of host defense, pathogen fitness, associated microbiome, or their combined effect. Here, we focused on pathogen response given the observation of high variability in pathogen population on resistant cultivar under elevated ozone compared to the ambient conditions. While susceptible cultivar showed dominance of a single pathogen genotype regardless of the environment, resistant cultivar supported co-occurrence of both pathogen genotypes under ambient environment, indicative of maintenance of heterogeneity as a strategy for adaptation. However, such heterogeneity was only evident during end season under elevated ozone. In addition to the altered strain dynamics, higher mutation rate, and presence of de novo parallel mutations in the pathogen population under elevated ozone during single season are suggestive of a plastic eco-evo response of pathogen population to adapt to the resistant cultivar under altered climate.

Introduction

Climate change accompanied by changes associated with modern agricultural practices have presented a threat of emerging novel pathogen lineages capable of compromising host resistance or expanding their host range. Resistance breakdown or erosion is a major concern in agricultural crops (Ka et al., 2006; Leach et al., 2001; Papaïx et al., 2018) and understanding the management of durability under future climatic conditions is a priority. Pathogen selection pressure triggers the dynamics of recognition avoidance and balancing fitness penalties, and in many cases, facilitating long-term maintenance of polymorphism (Bakker et al., 2006; Karasov et

al., 2014; Mauricio et al., 2003) in avirulence and qualitative resistance genes (Leach et al., 2001; Leonard & Czochor, 1980; Van der Plank, 1968; Vera Cruz et al., 2000). Quantitative resistance (polygenic), on the other hand, that manifests as a more continuous distribution of resistance phenotypes within a population (St Clair, 2010), is thought to evolve differently resulting in a continuous distribution of pathogen adaptation (Caffier et al., 2016; Corwin & Kliebenstein, 2017; St Clair, 2010). In comparison to qualitative resistance, quantitative resistance is thought to be durable. Climate-sensitivity has been noted in basal resistance (Cheng et al., 2013; Janda et al., 2019), qualitative resistance and quantitative resistance, with the exception of a few to be climate-resilient (Cohen et al., 2017). Climate sensitivity of pathogens during their interaction with the plants is unclear (Huot et al., 2017; Onaga et al., 2017; Velásquez et al., 2018) and unlike animal pathogens (Shapiro & Cowen, 2012), little is known about modulation of virulence in plant pathogens under abiotic stress. Both climatic shifts and biotic stress may have played an important role in complex evolutionary adaptations in plants and in shaping plant physiological and morphological traits (Bouda et al. 2022; Mitchell and Whitney 2018; Rauschkolb et al. 2022). While resurrection experiments may illuminate on these adaptations, increasing magnitude of extreme climate events still leave us with the unpredictability as to how plants will adapt to simultaneous stressors. This is especially true for agricultural systems where climate change, global trade and modernization have predisposed the systems to increased threat of pests (Ka et al. 2006; Godefroid et al. 2022; Burbank et al. 2022), leading to different selection pressures compared to those in the recent past (Meline et al. n.d.).

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avoidance and balancing fitness penalties, and in many cases, facilitating long-term maintenance of polymorphism (Bakker et al. 2006; Karasov et al. 2014; Mauricio et al. 2003a) in avirulence and qualitative resistance genes(Van der Plank 1968; Leonard and Czochor 1980; Vera Cruz et al. 2000; Leach et al. 2001). Quantitative resistance (polygenic), on the other hand, that manifests as a more continuous distribution of resistance phenotypes within a population(St Clair 2010), is thought to evolve differently resulting in a continuous distribution of pathogen adaptation(St Clair 2010; Caffier et al. 2016; Corwin and Kliebenstein 2017). In comparison to qualitative resistance, quantitative resistance is thought to be durable. Climate-sensitivity has been noted in basal resistance(Janda et al. 2019; C. Cheng et al. 2013), qualitative resistance and quantitative resistance[20,46,56], with the exception of a few to be climate-resilient(Cohen et al. 2017). Climate sensitivity of pathogens during their interaction with the plants is unclear (Velásquez, Castroverde, and He 2018b; Huot et al. 2017; Onaga et al. 2017) and unlike animal pathogens(Shapiro and Cowen 2012), little is known about modulation of virulence in plant pathogens under abiotic stress.

Genetic variation in plant pathogens has been studied extensively to characterize diversity between related species or among individuals of the same species (Moller and Stukenbrock, 2017; Wang et al. 2017). Rapid adaptation or host specialization in fungal plant pathogens resulting in highly dynamic genome architectures have been noted (Rouxel et al. 2011; van Dam 2016), also effectors as rapidly evolving genes. Many of these validated virulence factors. Examples of high intraspecific diversity at the population level in plant pathogens have been studied (Moller and Stuckenbrock, 2017). Rapidly evolving genomic regions enriched in transposable elements (TE), carrying clusters of virulence determinants, have been associated with pathogen's ability to adapt to host resistance or environmental fluctuations (Whisson et al. 2012, FRantzeskakis et al. 2019, Croll). Distinct genomic compartments were found to have different rates and modes of evolution

(Raffaele et al. 2010; Moller and Stuckenbrock 2017). Here we conducted in-depth sampling of nucleotide diversity from different genomic compartments with high resolution at the population genomic level. We applied population genetic analysis in the framework of metagenomic survey of plant pathogenic bacteria subjected to biotic and abiotic stress and identified genetic targets of selection. Previously, such studies have been conducted on isolate genomes (Grunwald et al. 2016; Badouin et al. 2017; Hartmann et al, 2018; Thilliez et al. 2019). Distribution and frequency of SNPs across genome can allow identification of genomic regions that have been targets of recent directional selection (Stephen 2016), some associated with virulence, host specificity (Hall et al. 2009, Poppe et al. 2015). Directional or positive selection will lead to the fixation of a single allele through a selective sweep of the allele conferring higher fitness. Under this scenario, polymorphism is transient, and one allele will dominate over the others eventually. On the other hand, some virulence factors show signs of balancing or diversifying selection in which alternative alleles are maintained within populations (as seen with some effectors, Brunner and McDonald 2018). This type of selection leads to the retention of seq diversity within populations. Allele frequency can vary over time depending upon variation of resistance factors present in the host, or type of selection pressures. In addition to variation, gene gain or less facilitated by transposable elements have been known to be important for rapid adaptation of fungal or oomycete pathogens and introducing genetic novelty in the population (Yoshida et al. 2016, Hartmann and Croll 2017, Tsuhima et al. 2019). Grandaubert et al. (2019) used population genomic dataset combined with statistical genetics approach to quantify rates of adaptation across genome of *Z. tritici*. They found high rates of adaptive substitutions in effector-encoding genes but these high rates did not correlate with TE presence or abundance. Instead they found role of sexual recombination in allowing rapid fixation of beneficial mutations. Pathogen ecology, epidemiology and virulence evolution differ

among quantitative and qualitative resistance (Fabre et al. 2012, Baucom and de Rode, 2011). Quantitative resistance is recommended as a solution to prevent yield losses and halts the accelerated clonal cycles responsible for the epidemics. Being a polygenic resistance, pathogen cannot overcome quantitative resistance as rapidly as R genes. Due to weaker selection pressure for overcoming quantitative resistance compared to R-gene mediated resistance, quantitative resistance is traditionally assumed to be more durable (McDonald and Linde 2002, Poland et al. 2009, St Clair 2010, Brown and Rant 2013). But empirical data on its efficiency and stability in the light of climatic fluctuations is lacking.

Under harsh conditions within a host or in the presence of antibiotics, it is suggested that genetic changes follow the repair of DNA double-strand breaks (DSBs) – these genetic changes constitute the beneficial or disadvantageous genetic diversity underlying an organisms ability to rapidly adapt to those conditions (Gusa and Jinks-Robertson 2019). Much like the durability of quantitative resistance within the host, the process of pathogen adaptation under selection by host pressure has been shown to transpire following the mutation of multiple loci, often not associated with host plant interactions (Mohd-Assaad, McDonald, and Croll 2018; Mohd-Assaad, McDonald, and Croll 2016). The pressure for pathogen adaptation has been shown to be variable however, as in certain conditions there seems to be an increased drive for or presence of genomic variation. In a diploid pathogen, large-scale genomic changes such as aneuploidy and loss of heterozygosity (LOH) were recorded following exposure to host environments with those mutations associated with infection in immunocompromised hosts being fewer in number and occurring less frequently than in immunocompetent hosts – additionally, following sequential passaging in immunocompetent hosts, time to 50% mortality of hosts was significantly lower, suggesting that host immunity-induced genomic changes increased virulence and facilitated a response to host

selection pressures (A. C. Smith, Morran, and Hickman 2022). Researchers Smith et al., 2022 suggested that the increased production of reactive oxygen species (ROS) and antimicrobial peptides (AMPs) in immunocompetent hosts induces genotypic plasticity and facilitates rapid evolution (A. C. Smith, Morran, and Hickman 2022). Not only can hosts produce ROS to combat pathogenic infection, but environmental ROS may also play a role in pressuring pathogens to adapt.

The strain-resolved metagenomics approach allowed us to track genetic dynamics along with ecological fluctuations within dominant pathogen, Xanthomonas. Such approach allows us to investigate intra-host dynamics of pathogen population with high resolution into genetic processes shaping its evolution during a single growing season. We are looking at gene-to-population level changes occurring in the pathogen in response to host defense and altered ozone levels. Elevated ozone did not influence disease severity levels on susceptible cultivar as previously noted (Bhandari et al. 2023). However, under elevated ozone levels, the resistant cultivar showed significantly higher disease severity scores compared to ambient environment. This increase was accompanied by high variation, ranging from 2-38% during mid-season, to 0-8% during end-season. Elevated ozone caused average increase in disease severity of 12% during mid-season and 2% during end-season on resistant cultivar compared to ambient environment (Figure 5-1.C) (Bhandari et al. 2023). The high variation in disease severity levels could be the result of variation in host plasticity, direct or indirect (mediated through host plasticity) effects of climate on pathogen infectivity or altered ecological interactions among pathogen genotypes. In this study, we focused on investigating the influence of altered ozone levels on pathogen population from gene to population level and whether ecological fluctuations and/ evolutionary modifications in pathogen population could explain the increase in disease severity observed on the resistant

cultivar under elevated ozone conditions. As coexistence of multiple genotypes (based on trade-offs or cross-feeding) is proposed as one of the mechanisms for adaptation of bacteria to environmental stressor, we hypothesized that adaptation to host defense on resistant cultivar will involve population heterogeneity. In order to test this hypothesis, we chose coinfection by two closely related lineages of emerging pepper pathogenic species, *X. perforans*, isolated during the recent field sampling (Newberry et al. 2019, 2023), namely, SC6 (strain AL65, isolated from susceptible pepper cultivar) and SC2 (strain AL22, isolated from the resistant pepper cultivar). Such co-inoculation approach allowed us to study the strain turnover as it happens over the course of the growing season, under the influence of host resistance and altered ozone environment. The population genetic methods applied to the high-resolution shotgun metagenome data allowed us to capture the population dynamics at the intra-subspecific level of phylogenetic resolution. Overall, our data provide understanding of how population dynamics both shape and are shaped by evolutionary processes.

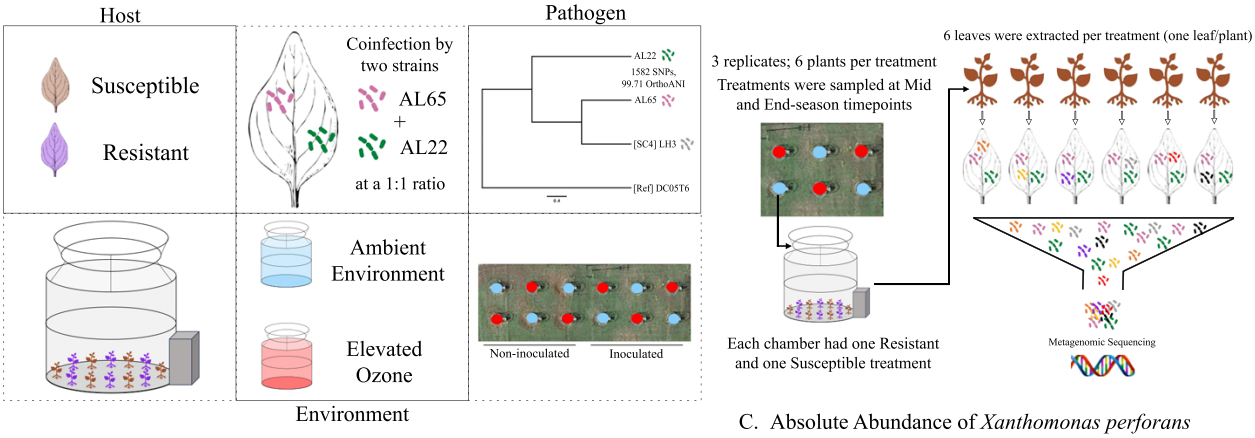
Results

Pathogen population dynamics was host-genotype-dependent and elevated ozone further altered strain dynamics on the resistant cultivar.

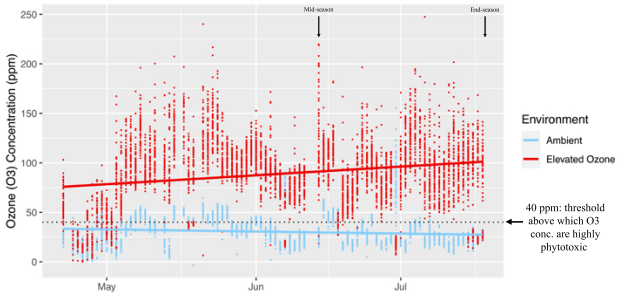
To obtain understanding of pathogen population response during adaptation to the resistant host and under altered ozone levels, we used strain-resolved metagenomics approach to temporally trace the frequencies of the co-inoculated pathogen lineages across the treatments. Although there was incidence of natural infection by another *X. perforans* lineage (SC4), this lineage did not increase in frequency during the growing season. The presence of host resistance had a significant effect on the relative and absolute abundance of AL65 (P=0.00395, Kruskal-Wallis) and AL22

(*P*=0.00395, Kruskal-Wallis) (Figure 5-1.D, E). Strain AL65 outperformed strain AL22 on the susceptible cultivar throughout the growing season, regardless of ozone levels. On the other hand, strain-level heterogeneity was observed on the resistant cultivar, although the host x environment interaction likely influenced the strain dynamics. Strain AL22 (originally isolated from resistant cultivar and thus can be assumed to be resistant cultivar adapted) was found to maintain higher population on the resistant cultivar under ambient environment during mid-season. Despite low absolute abundance of *Xanthomonas* during mid-season on the resistant cultivar, it is worth noting to observe the two strains with distinct niche preference, with AL65 being dominant under elevated ozone and AL22 under ambient conditions. By the end season, both strains co-existed on the resistant cultivar regardless of the environmental conditions (Figure 5-1.D, E).

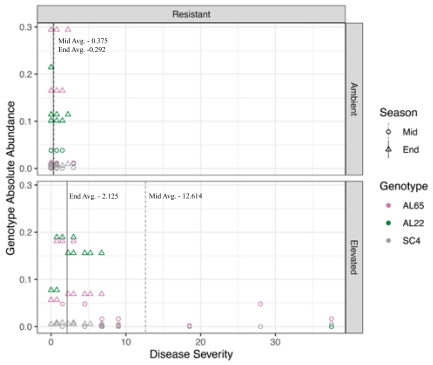
A. Experimental Setup and Methodology



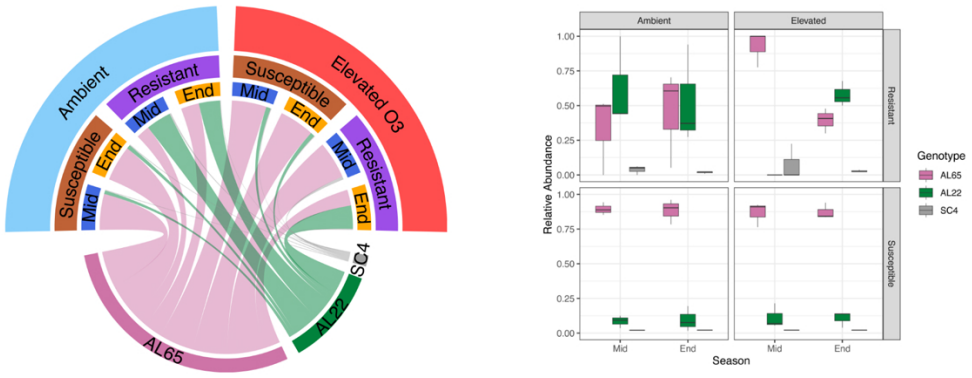
B. Ozone Levels (ppm) throughout the Season



C. Absolute Abundance of *Xanthomonas perforans* by Disease Severity



D. Relative Abundance of *Xanthomonas perforans* Strains



E. Absolute Abundance of *Xanthomonas perforans* Strains

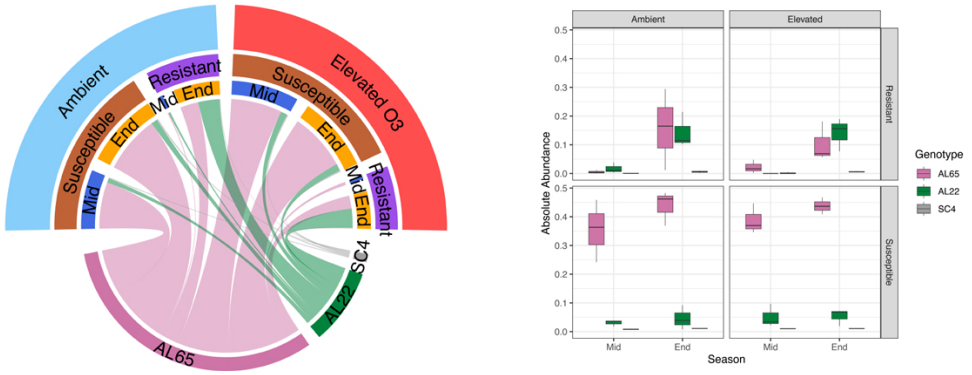


Figure 5-1. A general overview of this experiment.(A) We set up an experiment in which we inoculated two strains of *Xanthomonas perforans* at a 1:1 ratio onto either susceptible or resistant plants and exposed them to either ambient or elevated levels of ozone within open-top chambers. Out of 12 open-top chambers, 6 were non-inoculated acting as controls and 6 were inoculated; additionally, 6 were exposed to elevated ozone while the remaining 6 were not exposed. Each chamber contained 12 plants making up two treatments, half of which were a susceptible cultivar and half of which were resistant with each set being a treatment, respectively. At both a mid- and end-season timepoint, within each chamber, from the 6 plants within each treatment, 6 leaves were removed and their metagenomic DNA was extracted and sequenced. Genotypes AL22 and AL65 were sampled, as expected, however, evidence of natural infection by a representative of sequence cluster 4 (SC4) was found among the samples as well. Ozone levels throughout the season within the open-top chambers were on average 29.33 ppm for Ambient chambers and 87.65 ppm for Elevated Ozone chambers. Ozone levels above 40 ppm are considered to be highly phytotoxic; (B) Absolute abundance of *X. perforans* genotypes as compared to disease severity during the mid- and end-season sampling timepoints for the resistant cultivar under ambient versus elevated O3 levels. The mid- and end-season disease severity averages vary from ambient to elevated O3; (C) Relative abundance of *X. perforans* genotypes as averages in the leftmost circular chord diagram and as raw values in the rightmost barplot; (D) Absolute abundance of *X. perforans* genotypes as averages in the leftmost circular chord diagram and as raw values in the rightmost barplot.

Higher genetic differentiation in the pathogen population is observed on the resistant cultivar under elevated ozone.

Next, we asked whether genetic differentiation in the pathogen population may account for higher disease severity values seen under elevated ozone on the resistant cultivar since absolute abundance of *Xanthomonas* could not explain the observation (Figure 5-1). It is unclear how pathogen population adapts on the resistant cultivar carrying quantitative resistance genes, let alone under altered climatic conditions. Here, we hypothesized that adaptation to the stressors is reflected in higher genetic differentiation in the pathogen population. The stressors here are host defense (i.e. adaptation to resistant cultivar compared to the susceptible cultivar), or elevated ozone in either host background.

To evaluate pairwise genetic differentiation among populations recovered from different host genotypes and from ambient and elevated ozone environment, fixation index (F_{ST}) was calculated for each slide window (1 kbp) by Popoolation2. Average pairwise F_{ST} between pathogen populations recovered from susceptible cultivar under ambient and elevated ozone environments was the lowest of all pairwise comparisons. Comparing pathogen populations recovered from resistant cultivar under ambient and elevated ozone environment ($F_{ST(mid)}$: mean= 0.09922863; median=0.0801964) showed higher genetic divergence compared to that from susceptible cultivar under two environments ($F_{ST(mid)}$: mean=0.01708811; median=0). There was also influence of time of sampling on the level of genetic differentiation, with mid-season populations with higher pairwise F_{ST} compared to that of end-of season (Resistant $F_{ST(end)}$: mean= 0.11442667, median=0.07407407). However, adaptation to the resistant host under elevated ozone resulted in greater population divergence at the end of season. Next, we calculated within-host nucleotide diversity (π) and mean population mutation rate per site (θ) for each sample. Observations made

above for genetic differentiation were confirmed further by within-host nucleotide diversity values. We observed higher but variable within-host nucleotide diversity values and mean population mutation rate per site in the pathogen population recovered from resistant cultivar compared to susceptible cultivar irrespective of environmental conditions ($p\text{-value}_{\pi} = 0.000382$, $p\text{-value}_{\theta}$ = ns). End-season pathogen population recovered from resistant cultivar under elevated ozone conditions had greater within-host nucleotide diversity values and mutation rate than mid-season populations ($p\text{-value}_{\pi} < 0.0001$). Interestingly, nucleotide diversity for pathogen population recovered from resistant cultivar at the mid of season was significantly higher under ambient ozone conditions compared to the elevated environment ($p\text{-value}_{\pi} < 0.0001$, $p\text{-value}_{\theta}$ = ns), but there was no significant difference in the within-host nucleotide diversity values by the end-season (Figure 2B, 2D). This observation is in concordance with the finding of higher genetic differentiation on resistant cultivar when comparing across ambient and elevated ozone environments (Figure 5-2.A).

The mutation rate observed on the susceptible cultivar was average of ~ 8×10^{-05} and was unaffected by ozone levels. On the other hand, higher mutation rate was observed on resistant cultivar throughout the growing season, with ~ 7.17×10^{-4} per site during mid-season decreasing to 1.98×10^{-4} per site by the end of season. Elevated ozone led to a slightly lower mean mutation rate 6.12×10^{-4} per site during mid-season but maintaining overall higher levels of mutation rate of 4.22×10^{-4} per site by end season on the resistant cultivar, compared to that observed under ambient conditions (Figure 5-2.E).

Next, we measured the extent of within-host polymorphism in the pathogen population by identifying alleles displaying intermediate minor allele frequency (0.2-0.8) and classified the types of mutation as synonymous or nonsynonymous mutations (Figure 5-2.F). We compared

normalized SNV counts (relative to absolute abundance of *Xp* population) across treatments. Overall, higher number of SNVs were observed on the resistant cultivar compared to the susceptible cultivar under both environments ($p\text{-}value_{(\text{ambient})}$: mid< 0.0001 (****), end < 0.001 (**); $p\text{-}value_{(\text{elevated O3})}$:mid=0.04(*), end<0.0001 (****)). Further, the influence of elevated ozone was observed when comparing the pathogen population on resistant cultivar with the population on susceptible cultivars under ambient conditions (*p-value:* mid=0.001 (**); end<0.0001(****).

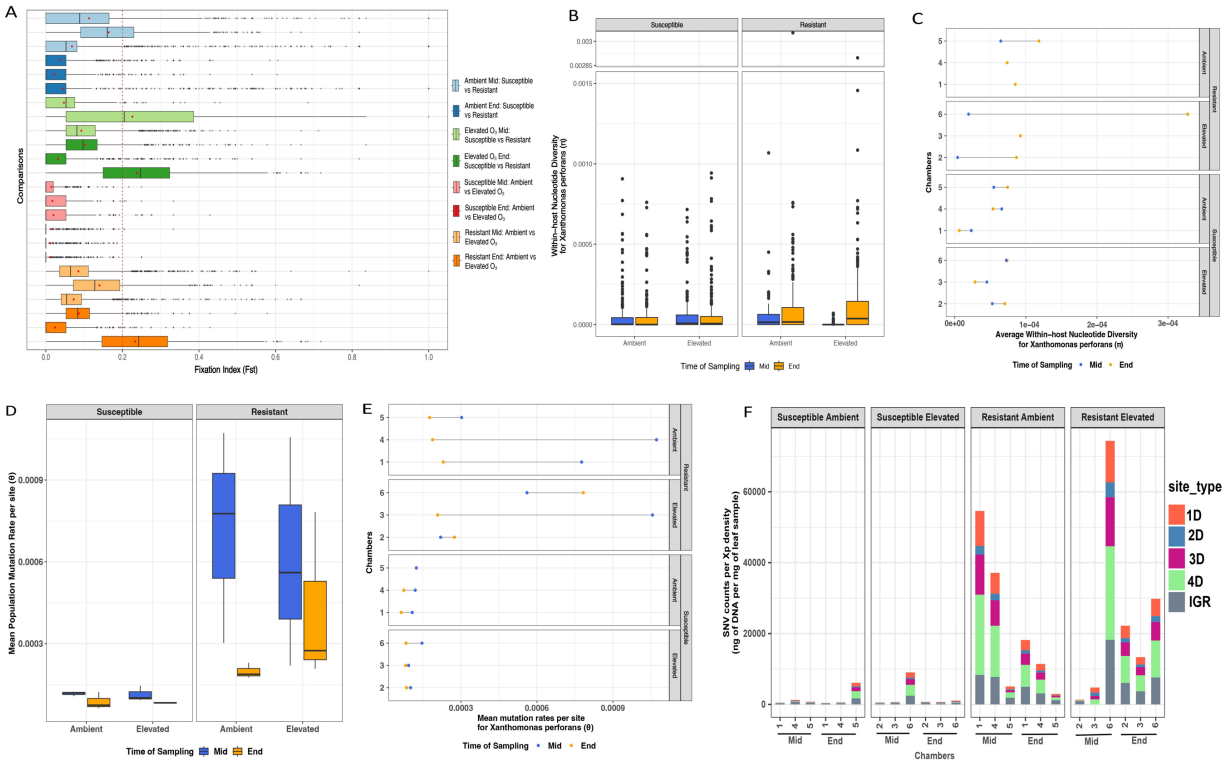


Figure 5-2. Genetic differentiation in the pathogen population. Pathogen population adapted to resistant cultivar under elevated O3 with higher genetic differentiation: (A) Boxplots showing Pairwise Host comparisons with Fst (Fixation Index) values (calculated using Poopulation2) for 1kbp window. The red color dashed line indicates (values greater than 0.2) threshold value

considered for differentiation; (B) Boxplots showing within-host nucleotide diversity of *Xanthomonas perforans (Xp)* population (π) together for different treatments; (C) line plot is showing average nucleotide diversity of *Xp* population (π) for each chamber; (D) boxplots with Mean mutation rates per site (θ) in *Xp* population; (E) line plot showing Mean mutation rates per site (θ) in *Xp* population for each chamber; (F) Barplots showing counts of different SNV (Single nucleotide variants) sites for each chamber with different types of mutations having minor allele frequency between 0.2 and 0.8. In figure, 1D, 2D, 3D and 4D are different types of mutations, where a site with 1D is one in which an amino acid change caused by nucleotide difference (non-synonymous), while a site with a 4D cannot be caused by any nucleotide difference (synonymous), and 2D & 3D indicates the either two or three possible changes, respectively can be tolerated, before an amino acid is altered (D. W. Chen and Garud 2022; Nayfach [2015] 2022).

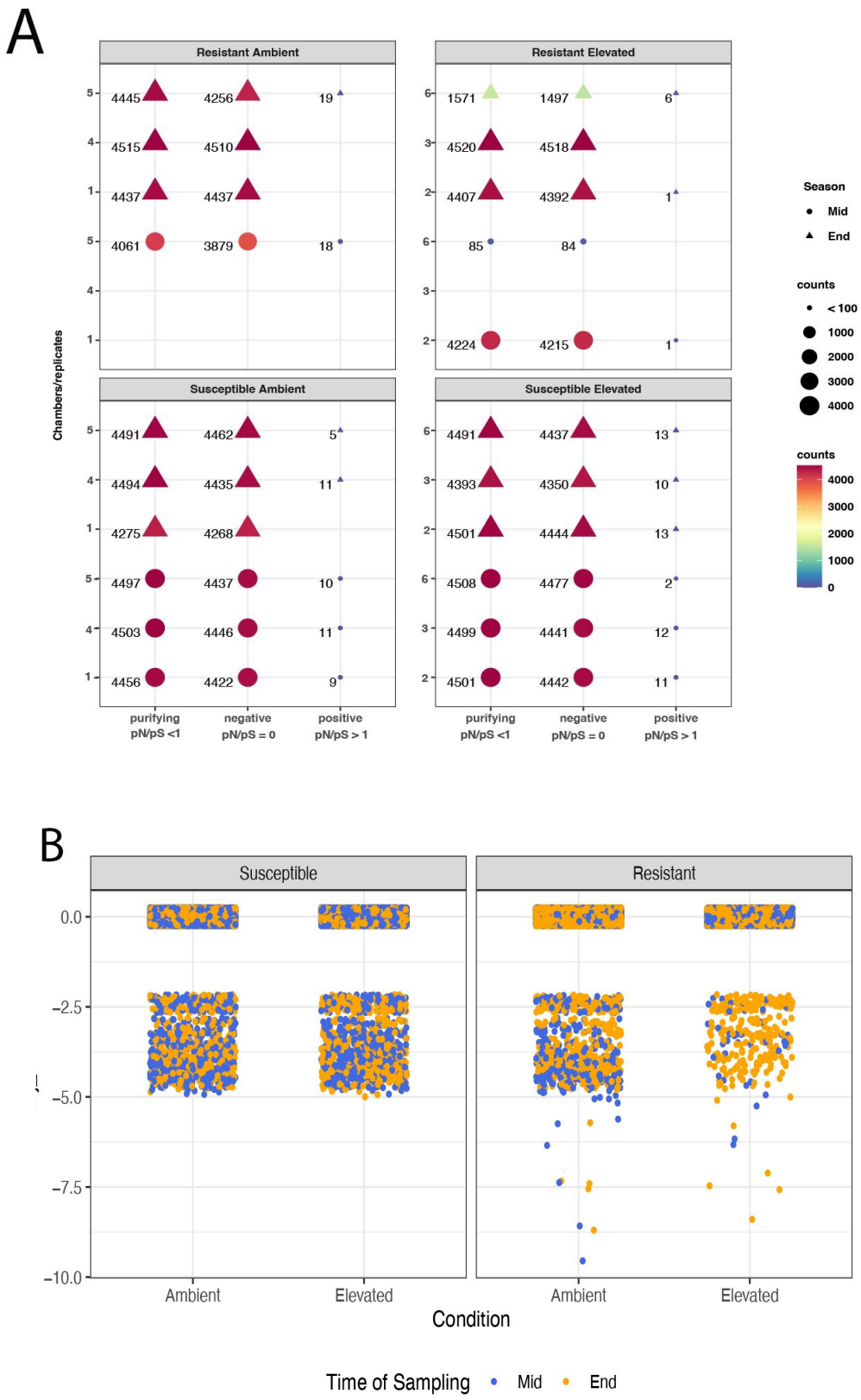

A
Resistant Ambient
Resistant Elevated
Susceptible Ambient
Susceptible Elevated
Chambers/replicates
purifying pN/pS <1
negative pN/pS = 0
positive pN/pS > 1
Season
Mid
End
counts
< 100
1000
2000
3000
4000
counts
4000
3000
2000
1000
0
B
Susceptible
Resistant
0.0
−2.5
−5.0
−7.5
−10.0
Ambient
Elevated
Condition
Time of Sampling
Mid
End

Figure 5-3. (A) The point graph shows the different selection types: negative selection or strong purifying selection (pN/pS = 0), positive selection (pN/pS >1) and purifying selection (pN/pS between 0 to 1); (B) Point graph presenting the Tajima's D values per gene across different treatments.

Pathogen population exhibited strong purifying selection in response to altered environment.

While the increased genetic differentiation observed within the *Xp* population may suggest the presence of a range of evolutionary processes, we delved deeper into our investigation to elucidate the precise mechanisms at work. Our hypothesis centered on the possibility of concurrent positive selection occurring within the pathogen population, in response to host defense on the resistant cultivar and under altered ozone conditions across different replicates. As a result, we scanned the data for the signatures of positive selection. To achieve this, we undertook an analysis of selective pressures within the genomic landscape, considering metrics such as Tajima's D and pNpS (Non-synonymous to Synonymous) values, aiming to uncover their contributions to the observed genetic differentiation. We detected significant differences in pN/pS ratios based on cultivar (p-values=1.35e-06, ****), season (p-value =1.578e-06, ****) and environment conditions (p-value =5.47e-09, ****). We observed that pN/pS ratios in populations from resistant cultivars exposed to elevated ozone conditions differed significantly from those in resistant cultivars under ambient conditions (p-value = 3.38E-05, ****) and susceptible cultivars under elevated ozone conditions (p-value = 2.31E-03, **) (Figure 5-3.A). Then, we categorized the genes according to their selection pressures, distinguishing between those under positive (pN/pS >1), negative (pN/pS = 0), and purifying selection (pN/pS > 1) based on the pNpS values. On an average, 81 percent of

the pangenome showed finite and defined pN/pS ratio values across all the samples. We observed 97% (mean= 4467, median=4495) and 66 % (mean = 3045, median = 4249.5) genes of pangenome exhibited purifying or stabilizing selection (pN/pS < 1) in population on resistant and susceptible cultivars respectively. Further, the proportion of genome under positive selection (pN/pS >1) was relatively small with an average of 0.2% (altered ozone) and 0.12 % (ambient) of the genes showing finite pN/pS values. Indicate how many of these under positive selection were parallel on susceptible cultivar. On the other side, ozone levels influenced the proportion of genome under purifying selection with 97% under ambient environment and 76% under altered ozone conditions (Figure 5-3.A). The proportion of genome under positive selection remained relatively small, with only 19 genes from one replicate under ambient conditions and 7 genes (1 in one replicate and 6 in second replicate) under elevated ozone under positive selection. Surprisingly, none of these genes were identified as common across replicates, indicating lack of parallelism (Table 5-1). Interestingly, we observed around ~ 98% of genes that were under purifying selection were under strong negative selective pressure (pN/pS = 0) in the pathogen population irrespective of conditions and host. It indicates the pathogen population maintained strong evolutionary conservation by maintaining their gene functions and natural selection was actively filtering out mutations which were deleterious or harmful for the pathogen population.

Next, we looked at significant differences in Tajima's D values. For instance, Tajima's D values were significantly different in the resistant host under elevated ozone conditions compared to the resistant cultivar under ambient conditions (p-value = 1.88E-02, *), as well as the susceptible cultivar under both ambient (p-value= 1.88E-02, *) and elevated ozone conditions (p-value = 1.03E-16, ****). The extremely negative values of Tajima's D in resistant cultivars by the end of

the season, regardless of conditions (falling below -2), indicate the presence of a selective sweep or rare alleles in the population (Figure 5-3.B).

Table 5-1. Genes parallel across chambers under positive selection (pN/pS >1 & Tajima'sD < 0).

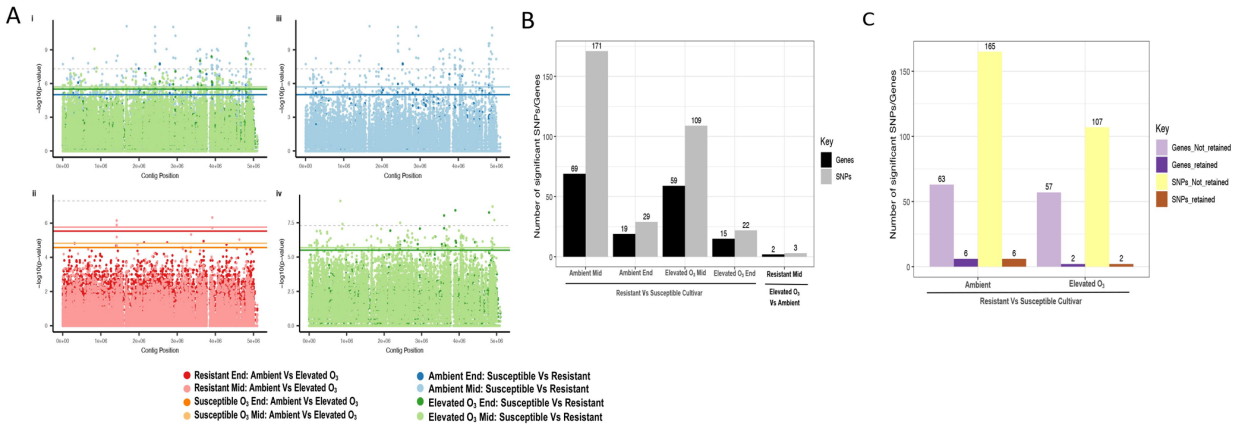
contig_gene	COG category	Description	Chamber Parallel	Treatment
SUPERPANG_100_length=177249_25	S	conserved protein (some members contain a von Willebrand factor type A (vWA) domain)	3	Susceptible Ambient Mid
SUPERPANG_47_length=204717_92	P	SulP family inorganic anion transporter	3	Susceptible Ambient Mid
SUPERPANG_59_length=357528_92	S	Acid phosphatase	3	Susceptible Ambient Mid
SUPERPANG_68_length=77131_43	K	Transcriptional regulator	3	Susceptible Ambient Mid
SUPERPANG_100_length=177249_90	KU	Transcriptional regulatory protein, C terminal	2	Susceptible Ambient Mid
SUPERPANG_47_length=204717_85	S	Protein of unknown function (DUF1684)	2	Susceptible Ambient Mid
SUPERPANG_47_length=204717_86	P	Sulfurtransferase	2	Susceptible Ambient Mid
SUPERPANG_54_length=138570_25	S	protein conserved in bacteria	2	Susceptible Ambient Mid
SUPERPANG_59_length=357528_229	S	transporter	2	Susceptible Ambient Mid
SUPERPANG_59_length=357528_95	K	Belongs to the sigma-70 factor family. ECF subfamily	2	Susceptible Ambient Mid
SUPERPANG_68_length=77131_30	E	aminopeptidase	2	Susceptible Ambient Mid
SUPERPANG_59_length=357528_92	S	Acid phosphatase	2	Susceptible Ambient End
SUPERPANG_68_length=77131_43	K	Transcriptional regulator	2	Susceptible Ambient End
SUPERPANG_47_length=204717_92	P	SulP family inorganic anion transporter	3	Susceptible Elevated Mid
SUPERPANG_100_length=177249_25	S	conserved protein (some members contain a von Willebrand factor type A (vWA) domain)	2	Susceptible Elevated Mid
SUPERPANG_100_length=177249_90	KU	Transcriptional regulatory protein, C terminal	2	Susceptible Elevated Mid
SUPERPANG_47_length=204717_85	S	Protein of unknown function (DUF1684)	2	Susceptible Elevated Mid

SUPERPANG_54_length=138570_25	S	protein conserved in bacteria	2	Susceptible Elevated Mid
SUPERPANG_59_length=357528_229	S	transporter	2	Susceptible Elevated Mid
SUPERPANG_59_length=357528_92	S	Acid phosphatase	2	Susceptible Elevated Mid
SUPERPANG_59_length=357528_95	K	Belongs to the sigma-70 factor family. ECF subfamily	2	Susceptible Elevated Mid
SUPERPANG_68_length=77131_27	J	Belongs to the class IV-like SAM-binding methyltransferase superfamily. RNA methyltransferase TrmH family	2	Susceptible Elevated Mid
SUPERPANG_68_length=77131_30	E	aminopeptidase	2	Susceptible Elevated Mid
SUPERPANG_68_length=77131_43	K	Transcriptional regulator	2	Susceptible Elevated Mid
SUPERPANG_100_length=177249_19	G	Catalyzes the transfer of a two-carbon ketol group from a ketose donor to an aldose acceptor, via a covalent intermediate with the cofactor thiamine pyrophosphate	3	Susceptible Elevated End
SUPERPANG_100_length=177249_90	KU	Transcriptional regulatory protein, C terminal	3	Susceptible Elevated End
SUPERPANG_47_length=204717_85	S	Protein of unknown function (DUF1684)	3	Susceptible Elevated End
SUPERPANG_47_length=204717_92	P	SulP family inorganic anion transporter	3	Susceptible Elevated End
SUPERPANG_54_length=138570_25	S	protein conserved in bacteria	3	Susceptible Elevated End
SUPERPANG_59_length=357528_92	S	Acid phosphatase	3	Susceptible Elevated End
SUPERPANG_59_length=357528_95	K	Belongs to the sigma-70 factor family. ECF subfamily	3	Susceptible Elevated End
SUPERPANG_68_length=77131_27	J	Belongs to the class IV-like SAM-binding methyltransferase superfamily. RNA methyltransferase TrmH family	3	Susceptible Elevated End
SUPERPANG_68_length=77131_43	K	Transcriptional regulator	3	Susceptible Elevated End
SUPERPANG_100_length=177249_25	S	conserved protein (some members contain a von Willebrand factor type A (vWA) domain)	2	Susceptible Elevated End
SUPERPANG_47_length=204717_86	P	Sulfurtransferase	2	Susceptible Elevated End
SUPERPANG_59_length=357528_229	S	transporter	2	Susceptible Elevated End
SUPERPANG_68_length=77131_30	E	aminopeptidase	2	Susceptible Elevated End

Signatures of parallel evolution in the pathogen population when subjected to different host genotypes and in presence of altered environment

Above observations of higher genetic differentiation and higher within-host nucleotide diversity values in the pathogen population recovered from resistant cultivar compared to susceptible cultivar led us to further examine SNPs differentiating across host genotypes and across ozone levels and evaluates the signatures of parallel evolution i.e. SNPs observed independently across three replicates. A Cochran-Mantel-Haenszel (CMH) test to assess the parallelism was conducted. Results from Manhattan plot shows that there are many significant SNPs when we compare the population from the Resistant vs Susceptible cultivar under both ambient and elevated ozone environment conditions with a Bonferroni-corrected threshold. However, there are only few significant SNPs comparing populations from resistant cultivars under ambient and elevated ozone conditions (Figure 5-4.A). In the mid-season, we identified 171 significant SNPs (spanning 69 genes) that were distinctly observed when comparing pathogen populations from resistant and susceptible cultivars under ambient conditions and 109 SNPs (spanning 59 genes) differentiating resistant and susceptible cultivars under elevated ozone conditions. By the end of the season, these numbers dropped to just 29 (spanning 19 genes) and 22 (spanning 15 genes), respectively (Figure 5-4.B). Further, there were only three SNPs (spanning 2 genes) identified as significantly different across elevated and ambient ozone conditions in pathogen population recovered from resistant cultivar. Next, we examined which of these above identified differentiating SNPs and associated SNP spanning genes were consistent across seasons i.e. differentiating SNPs identified in comparisons that were retained over the season. We found only six SNPs (spanning 6 genes) retained by the end season comparing resistant and susceptible cultivars under ambient conditions, while majority (165 SNPs) were not retained by the end season i.e. reverted back to the original

allele. Under elevated ozone conditions, we discovered two SNPs (spanning 2 genes) common across the pathogen population on resistant and susceptible cultivar (Figure 5-3.C). Surprisingly, we did not find any common SNPs or genes that were retained across seasons when comparing different environmental conditions. Genes in response to host defense under ambient conditions were annotated as ribosomal protein S6--L-glutamate ligase, molybdenum transport system permease protein, transketolase 1, a putative membrane protein, ribonuclease T, and Molybdenum cofactor guanylyltransferase. The genes annotated as Molybdenum cofactor guanylyltransferase and intergenic region were found significantly associated with host adaptation under elevated ozone conditions (Figure 5-3.D). Multiple SNPs were found in these two genes under both ambient and elevated ozone conditions across the season, suggesting their importance for host adaptation. Besides, a SNP in intergenic region was found only under elevated ozone.



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Population_comparisons	CHR	Contig_position	Ann	p-value
Ambient: Resistant Vs Susceptible	SUPERPANG_62_length=73671	4926	Ribosomal protein S6--L-glutamate ligase	1.46401E-07
	SUPERPANG_47_length=204717	109155	Ribonuclease T	1.08655E-06
	SUPERPANG_54_length=138570	65507	Molybdenum cofactor guanylyltransferase	3.04232E-06
	SUPERPANG_100_length=177249	4753	Molybdenum transport system permease protein ModB	1.27153E-06
	SUPERPANG_100_length=177249	89923	Putative membrane protein	2.53324E-06
	SUPERPANG_100_length=177249	20611	Transketolase 1	7.67784E-06
Elevated O3: Resistant Vs Susceptible	SUPERPANG_47_length=204717	117319	Interegenic Region	1.31111E-06
	SUPERPANG_54_length=138570	65508	Molybdenum cofactor guanylyltransferase	1.15442E-06

Figure 5-4. (A) Manhattan plot estimated for single SNP GWAS (Genome-Wide Association Study) results for different comparisons of population structure. The grey color dashed line is the default GWAS threshold of 5 x 10^{-8}. The coloured lines present the Bonferroni-corrected threshold, which is 0.05 divided by the number of SNPs in the summary statistics. (B) Barplot indicates the total number of SNPs and their associated genes were parallel across different comparisons. (C) Barplot showing the total number of SNPs and their associated gene counts persisted by the end season or counts which revert back to orginal state or lost from pathogen population by end season; (D) Table presenting all the SNPs associated genes with annotations and locations in genome which were persistent across season.

Genetic differentiation observed across cultivars and ozone conditions is due to standing genetic variation as well as de novo mutations.

The greater genetic divergence observed on the resistant cultivar may be attributable to the co-existence of both pathogen lineages i.e. standing genetic variation and/ de novo mutations. Indeed, results on strain dynamics indicated presence of both pathogen lineages during both mid and end season samples recovered from resistant cultivar under ambient conditions. In this case of oligo-colonization of the host by known lineages as well as in case of pathogen population undergoing fluctuation in lineage frequencies under elevated ozone on resistant cultivar, the contribution of evolutionary modifications cannot be ruled out. Single nucleotide variants (SNVs) and differences in gene content that we identified in above analyses can arise as a result of shift in lineage frequency as well as evolutionary changes occurring in response to stressors. Here we attempted to distinguish these two scenarios by tracking SNVs in the shotgun metagenome data. Our experimental design involving co-inoculation of two closely related lineages of pathogen (1582 SNV apart) allowed us to assign the alleles to the parent genomes and tease apart those arising during the course of growing season. Apart from strainEST method used above that relies on reference strain mapping approach, we analyzed the shotgun metagenome data for confirming oligo-colonization of resistant cultivar by analyzing minor allele frequency distribution patterns. The distribution of minor allele frequency (MAF) can be used to identify "strains" i.e. presence of distinct sub-populations within a larger population (ref). If the MAF < 0.1, indicated the presence of one dominant strain/pathogen lineage. The distribution suggests that two predominant strains exist, when MAF > = 0.5. If the MAF falls within the range of 0.1 to 0.5, it suggests the possible presence of more than two strains. The MAF < 0.1 for pathogen population recovered from susceptible cultivar under both environments in majority of the replicates confirmed the

dominance of one strain in population. However, in one of the replicates, we observed MAF close to 0.2, indicating existence of 2 or more strains. The low abundance of *Xanthomonas* in mid-season on resistant cultivar led to undetectable patterns in two replicates for ambient conditions. MAF values of ~0.3-0.4 in populations from resistant cultivar under ambient conditions during end season may indicate presence of two or more strains (Figure 5-5). Resistant cultivar under elevated ozone supported one strain (MAF<0.1) during mid-season, which shifted to MAF =0.5 by the end season, indicative of existence of two strains (Figure 5-6.A). Overall, these results confirm the observation of shift in strain dynamics on resistant cultivar when subjected to different ozone conditions (Figure 5-1.D).

Next, to assess for the presence of de novo mutations arising in the pathogen populations over the course of a single growing season during adaptation onto the resistant cultivar and the altered environmental conditions, we examined for the selective sweep. Alleles sweeping in the population were identified by looking for those minor alleles with MAF < 0.2 (less than in 20% of population) i.e. rare alleles observed during mid-season but those that shifted to MAF > 0.8, becoming major allele by end season. These rare sweeping alleles could be potentially adaptive. Interestingly, we did not find any such alleles in the susceptible cultivar irrespective of environmental conditions. In addition, we observed only 5 allelic sites (in only one replicate) in pathogen in response host defense (on resistant cultivar) under ambient conditions. However, in response to resistant cultivar and altered ozone conditions, pathogen population had a total of 397 sweeping alleles. Intriguingly, we observed 47 of these sweeping alleles across two replicates and only 4 observed as parallel sweeping alleles across all three replicates. All these four sweeping alleles spanned a single gene, coding for acid phosphatase and were mapped to AL65, indicating that these sweeping alleles were observed due to a strain turnover i.e. increased abundance of

AL65. Sweeping alleles observed in parallel across two or three chambers were in the genes encoding chemotaxis proteins, acetyltransferases, type II secretion systems, TonB-dependent receptors (as listed in the annotated sites table, Figure 5-6.C). These sweeping alleles were matched to AL65 and AL22 to identify novel SNVs that were observed to be arising in the population. We identified 14 novel single nucleotide variants (SNVs) that did not match any of our inoculated or naturally occurring contaminant strains. Out of 14 alleles, 4 were present in two replicates. These sites were further associated with one unknown gene, spanning three intergenic regions. These findings suggest that many of the genomic changes observed as differentiating across hosts or ozone conditions were due shift in the abundance of lineages (strain turnover) but there was a minor contribution of de novo mutations occurring in these populations during adaptation onto resistant cultivar and when subjected to elevated ozone.

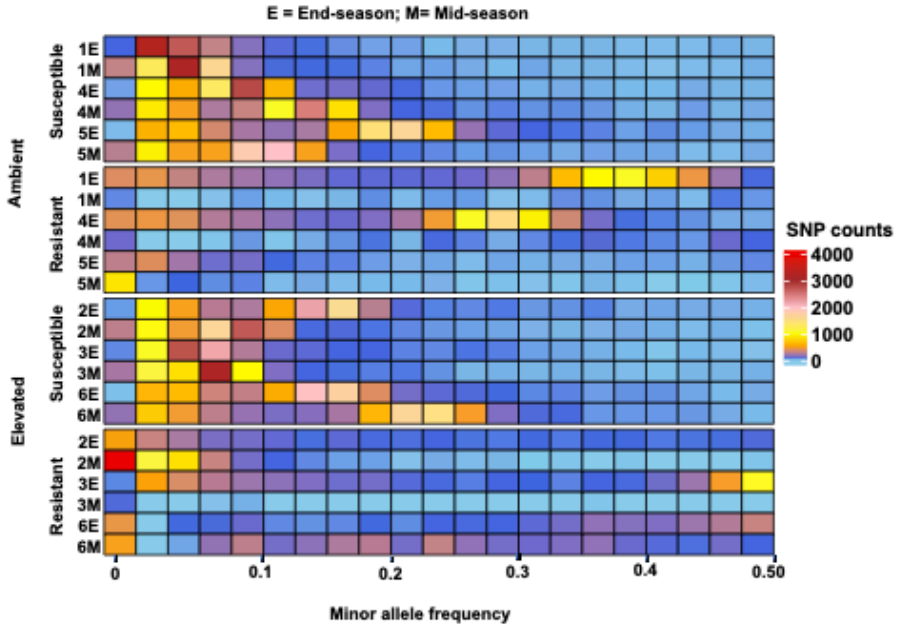

Figure 5-5. Heatmap presenting the counts of different minor alleles with frequency from 0 to 0.50 in the pathogen population among different treatments.

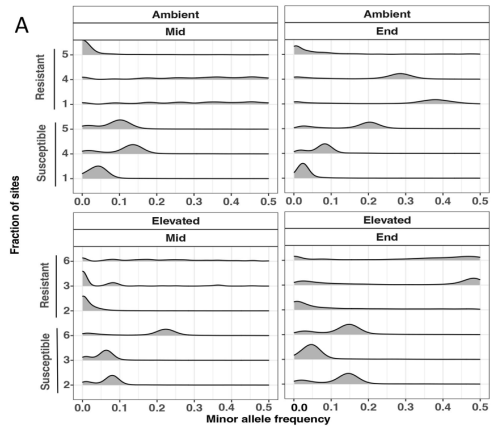

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Table: Parallel minor alleles in Xp population in resistant cultivar under elevated ozone condition

Parallel Across	site_id	gene_id	gene description	ref pos	Mutation type	AL22 min allele	AL65 maj allele	LH3 maj allele
Three Chmabers	3300699	SUPERPANG_02958	Acid phosphatase	115227	4D (syn)		TRUE	TRUE
	3300702	SUPERPANG_02958	Acid phosphatase	115230	4D (syn)		TRUE	TRUE
	3300708	SUPERPANG_02958	Acid phosphatase	115233	4D (syn)		TRUE	TRUE
	2644249	SUPERPANG_02958	Acid phosphatase	115239	4D (syn)		TRUE	TRUE
Two Chambers	2279623	SUPERPANG_00122	acetyltransferase	155401	3D	TRUE		
	2968563	SUPERPANG_02182		3679	4D (syn)	TRUE		
	4266431	SUPERPANG_02695	TonB-dependent receptor	31692	4D (syn)	TRUE		
	519409	SUPERPANG_03681	Belongs to the resistance-nodulation-cell division (RND) (TC 2.A.6) family	69763	1D (non-syn)	TRUE		
	579871	IGR		21745		TRUE		
	1833447	IGR		39250		TRUE		
	1833494	IGR		174379		TRUE		
	3300705	IGR		174426		TRUE		
	3300713	SUPERPANG_02958	Acid phosphatase	115236	4D (syn)		TRUE	TRUE
	3300714	SUPERPANG_02958	Acid phosphatase	115244	1D (non-syn)		TRUE	TRUE
	3300741	SUPERPANG_02958	Acid phosphatase	115245	4D (syn)		TRUE	TRUE
	3342295	SUPERPANG_02958	Acid phosphatase	115272	3D		TRUE	TRUE
	3988584	SUPERPANG_02982	In addition to polymerase activity, this DNA polymerase exhibits 5'-3' exonuclease activity	156826	3D		TRUE	TRUE
	171198	SUPERPANG_03491	Belongs to the cysteine synthase cystathionine beta-synthase family	63383	4D (syn)		TRUE	TRUE
	171368	IGR		40			TRUE	TRUE
	190303	IGR		210			TRUE	TRUE
	2787409	IGR		19145			TRUE	TRUE
	2787411	IGR		118156			TRUE	TRUE
	2787412	IGR		118158			TRUE	TRUE
	2787414	IGR		118159			TRUE	TRUE
	3433889	IGR		118161			TRUE	TRUE
	3433892	IGR		248420			TRUE	TRUE
	3433899	IGR		248423			TRUE	TRUE
	3535299	IGR		248430			TRUE	TRUE
	3535302	IGR		349830			TRUE	TRUE
	3988807	IGR		349833			TRUE	TRUE
	3988808	IGR		63606			TRUE	TRUE
	3988810	IGR		63607			TRUE	TRUE
	2716662	IGR		63609			TRUE	TRUE
	2716664	SUPERPANG_02480	type IV pilus biogenesis stability protein PilW	47409	1D (non-syn)		TRUE	
	2792115	SUPERPANG_02480	type IV pilus biogenesis stability protein PilW	47411	4D (syn)		TRUE	
	3376648	SUPERPANG_02532	Type VI secretion system effector, Hcp	122862	2D		TRUE	
	3376870	SUPERPANG_03010	Catalyzes the oxidation of glucose 6-phosphate to 6-phosphogluconolactone	191179	4D (syn)		TRUE	
	3376879	SUPERPANG_03010	Catalyzes the oxidation of glucose 6-phosphate to 6-phosphogluconolactone	191401	4D (syn)		TRUE	
	3376883	SUPERPANG_03010	Catalyzes the oxidation of glucose 6-phosphate to 6-phosphogluconolactone	191410	3D		TRUE	
	3376900	SUPERPANG_03010	Catalyzes the oxidation of glucose 6-phosphate to 6-phosphogluconolactone	191414	1D (non-syn)		TRUE	
	280362	SUPERPANG_03010	Catalyzes the oxidation of glucose 6-phosphate to 6-phosphogluconolactone	191431	3D		TRUE	
	3373384	SUPERPANG_04486	Catalyzes the synthesis of the hydroxymethylpyrimidine phosphate (HMP-P) moiety of thiamine from aminoimidazole ribotide (AIR) in a radical S-adenosyl-L-methionine (SAM)-dependent reaction	109204	4D (syn)		TRUE	
	4870845	IGR		187915			TRUE	
	579878	SUPERPANG_04197		82048	1D (non-syn)			
	3443167	IGR		39257				
	3443168	IGR		257698				
	3300696	IGR		257699				

Figure 5-6. Evolutionary modifications with strain replacement exhibited in pathogen population in resistant host under elevated ozone. (A) Density plots showing the distribution of minor alleles in *Xp* population across different chambers distribution of SNV counts with minor allele frequency; (B) Figure presenting the number of alleles which increased in frequency from < 0.2 to > 0.8 (from minor to major allele) for *Xp* population in resistant cultivar under elevated O_3; (C) Table presenting different allelic sites parallel across two and three replicates in the treatment of resistant cultivar under elevated O_3 with associated gene annotations, genome and mutation types. Here 1D, 2D, 3D and 4D are different types of mutations, where a site with 1D is one in which an amino acid change caused by nucleotide difference (non-synonymous), while a site with a 4D cannot be caused by any nucleotide difference (synonymous), and 2D & 3D indicates

the either two or three possible changes, respectively can be tolerated, before an amino acid is altered (D. W. Chen and Garud 2022; Nayfach [2015] 2022).

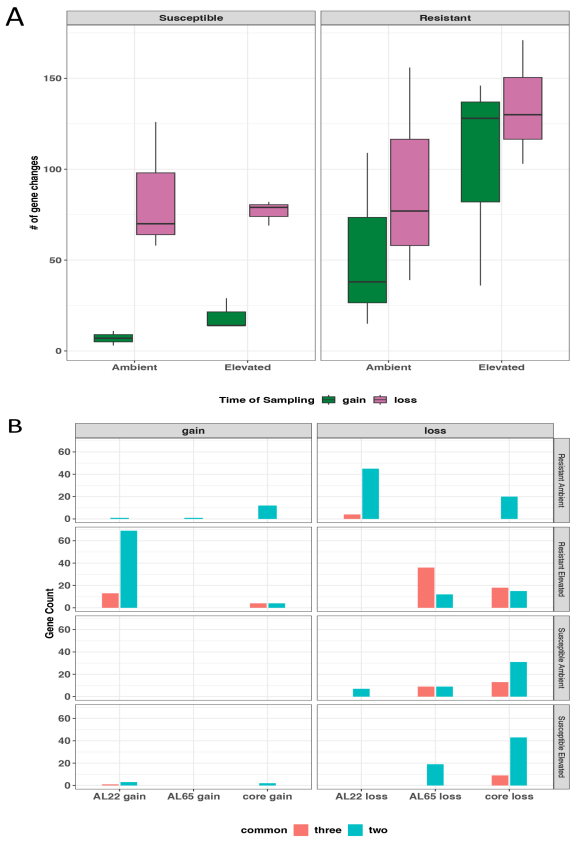
Higher rate of gene flux events was observed in the pathogen population during adaptation to resistant cultivar.

Another mechanism to adapt to different niches, pathogens can undergo gene gain or loss. To illustrate this aspect of evolutionary change, we quantified alterations in the gene pool that reflect fluctuations in pangenome size. We hypothesized that selection pressure will drive changes in pathogen gene pool by promoting the mechanisms of gene acquisition or loss to enhance pathogen fitness, and thus, we will observe more gene gain/loss events in pathogen populations subjected to combined stress i.e. adaptation to resistant cultivar under elevated ozone. In this context, we defined “core genes” as those shared among two of the inoculated strains, AL65 and AL22, while “accessory genes” are unique to each strain. Here, we defined "gene gain" as the situation where a gene was missing in the mid-season in population but reappeared at the end, and "gene loss" as the situation where a gene was present in the mid-season but was lost at the end-season. We observed a higher rate of gene flux in the pathogen population within resistant cultivar particularly under elevated ozone conditions, however, there were no differences in pathogen population from susceptible cultivars irrespective of environmental conditions (Figure 6A). We identified a total of 90 gene gain and 81 gene loss events in resistant cultivar under elevated ozone, out of which primarily gene gain events occurred in *Xp* AL22 and losses were in the *Xp* AL65 genome (Fig 5-7.B). In contrast, the population in the susceptible cultivar was found to be losing the core genome components under both conditions (Figure 5-7.C).

To further understand functional significance of these gain/loss events across the parallel genes among 2 and 3 chambers among different treatments, we used the Cluster of Orthologous Groups (COG)-based functional profiling. The pathogen population recovered from the susceptible cultivar under ambient conditions had lost genes of COGs [C] (Energy production and conversion), [MU] (Cell wall/ membrane/ Envelope biogenesis; Intracellular Trafficking, secretion and vesicular transport), [P] (Inorganic ion transport and metabolism). But population under elevated ozone conditions was observed to gain COGs [K] (Transcription), [L] (Replication, recombination and repair), [M] (Cell wall/ membrane/ Envelope biogenesis), and loss of [O] (Post-translational modification, protein turnover, chaperones (Figure 5-7.C).

On the other hand, when comparing the two populations from resistant cultivars, we found under ambient conditions, functions associated with COG [K] (Transcription), [L] (Replication, recombination and repair), [M] (Cell wall/ membrane/ Envelope biogenesis), [U] (Intracellular trafficking, secretion and vesicular transport), [E] (Amino acid transport and metabolism) were lost, but these functions were gained under elevated ozone conditions. Additionally, COG [C] (Energy production and conversion), [F] (Nucleotide production and metabolism), [O] (Post-translational modification, protein turnover, chaperones), [P] (Inorganic ion transport and metabolism), [Q] (Secondary metabolites biosynthesis, transport and catabolism), [MU] (Cell wall/ membrane/ Envelope biogenesis; Intracellular Trafficking, secretion and vesicular transport), and [PT] (Inorganic ion transport and metabolism; Signal Transduction mechanisms) were also gained under elevated ozone conditions in resistant cultivar. Moreover, functions associated with COG [NU] (Cell motility; Intracellular Trafficking, secretion and vesicular transport) and [T] (Signal Transduction mechanisms) were gained in the population under resistant cultivars under ambient conditions and were lost from elevated ozone conditions (Figure 5-7.C).

Remarkably, COG [V] was consistently lost from the population under all conditions, except when the pathogen was subjected to both host defense and environmental stress simultaneously. Overall, COG [K] (transcription) and [L] (function unknown) appear to be crucial for the population's adaptation to elevated ozone stress, as they were consistently gained by the population, regardless of the cultivar. In addition to this, pathogen population irrespective of cultivar and conditions, specifically, in resistant cultivar under elevated ozone conditions, we observed both gain and loss in the functions categorized as [S] (Function unknown) and *NA* (Hypothetical proteins and not found), which necessitate further investigation to understand functional significance (Figure 5-7.C).



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	Resistant								Susceptible							
	Ambient				Elevated O3				Ambient				Elevated O3			
	gain		loss		gain		loss		gain		loss		gain		loss	
parallel	two	three	two	three	two	three	two	three	two	three	two	three	two	three	two	three
Total	14	-	65	4	73	17	27	54	-	-	47	22	5	1	62	9
AL22	1		45	4	69	13					7		3	1		
AL65	1						12	36			9	9			19	
core	12		20		4	4	15	18			31	13	2		43	9
Total	14		69		90		81		0		69		6		71	
COG categories																
C					2	1						1				
F					2			1								1
G			2					1			1					1
GM			2				1	1			3				2	1
H			1					1			1				1	
K	1		1		6	1		1					1			
L			4		9	2							1			
M			1		2		1	2			4	1			6	
NU	1				1		3	3			1	4			6	
O					1			1							1	
S	3		9		7	3	6	4			4	4			4	3
U			4		1	2	1	3			5	1			4	1
D															1	
E			1		4											
I							1									
IQ			2				2				1				1	
IU			1													
J			1					1			1				1	
KT			1													
MU					1						1					
P					10						6					
PT					1											
Q					1											
T	1						1									
V			1								1				1	
NA	8	0	34	4	25	8	11	35	0	0	18	11	3	1	34	2

Figure 5-7. Gene flux in the pathogen population. (A) Boxplot showing the number of gene gain (absent during mid-season and present by end season in the pathogen population) and gene loss (present during mid-season and lost by end of season) across all the chambers. (B) Barplots presenting number of gene changes common among two and three chambers/replicates of different treatments. (C) Table showing the COG (Cluster of Orthologous Groups) based functional profiling of gene changes common in two and three chambers. The description of COG categories are: C = Energy production and conversion; F= Nucleotide metabolism and transport; G= Carbohydrate metabolism and transport; GM = Carbohydrate metabolism and transport & Cell wall/membrane/envelop biogenesis; H= coenzyme metabolism; K = transcription; L= replication and repair; M = Cell wall/membrane/envelop biogenesis; NU= cell

motility and Intracellular trafficking and secretion; O = Post-translational modification, protein turnover, chaperone functions; S = Function Unknown; U = Intracellular and trafficking and secretion; D = Cell cycle control and mitosis; E= Amino acid metabolism and transport; I=Lipid metabolism; IQ = Lipid metabolism & Secondary structure; IU = Lipid metabolism & Intracellular and trafficking and secretion; J = Translation; KT = transcription & Signal transduction; MU= Cell wall/membrane/envelop biogenesis & Intracellular and trafficking and secretion; P = Inorganic ion transport and metabolism; PT = Inorganic ion transport and metabolism; Q = Secondary Structure; T = Signal Structure; V = Defense mechanisms; NA= no hits found or Hypothetical protein with blast results.

Methodology

Relative Abundance

To understand factors which can alter intraspecific strain dynamics over the course of the growing season in pepper plants coinfected with two closely related strains: *Xanthomonas perforans* (*Xp*) AL65 and AL22, StrainEst was used to map single nucleotide variants (SNVs) of the metagenomic 2021 mid-season and end-season reads to a custom reference SNV profile mapped to the selected sequence representative *Xp* 91-118 (Albanese et al. 2017). Overall, the custom reference SNV profile contained strains representative of six sequence clusters (SC) within *Xp*: *Xp* AL65 (SC6), AL22 (SC2), AL33 (SC5), AL57 (SC3), GEV993 (SC1), and LH3 (SC4) (Newberry et al., 2019) - by opting to not only profile the coinfected strains, AL65 and AL22, in our StrainEst analysis, but also strains within the remaining four SCs, we could additionally evaluate any presence natural infection in our samples as well as our primary strains. In comparing the frequency of those SNVs present between the custom profile that was created and the metagenomic reads, StrainEst detected

the presence or absence of AL65, AL22, and any natural infection along with their relative abundances (RA) in the coinfected pepper plants.

Note: While the presence of those strains which were not inoculated in the plants would represent presence of the entire SC, since the identities of the strains we inoculated are known, we are considering presence of those strains to be presence of the strain itself rather than the SC. Distinguishment between either strain and presence of another strain within the same SC was not considered.

Note: Statistical significance was denoted by a p-value of <.05.

Non-redundant pangenome of Xp inoculated strains

Non-redundant pangenome of AL65 and AL22 was generated by the program SuperPang (v0.9.4beta1) (Puente-Sánchez et al. 2022), which was used as reference to map the metagenomic reads.

Identification of Blacklisted genes

Initially, we constructed a database using MIDAS, utilizing the non-redundant pangenome of *Xp* strains AL65 and AL22. This pangenome was generated by the SuperPang program, which provided us with the necessary files (.fna, .fasta, .ffn) for building the downstream analysis database. We created the ".genes" files using the conda-installed version of csvtk (version unspecified). Subsequently, we established the database using MIDAS (v1.3.2) (Nayfach [2015] 2022), requiring the following tools: hmmer-3.3.2, vsearch/2.14.1, and anaconda/3-2020.11.

We executed the "run_midas.py species" command to identify the predominant species in the samples, utilizing the default MIDAS database. This database comprised 31,007 bacterial reference genomes organized into 5,952 species groups and relies on a 95% genome-wide average nucleotide identity (ANI). From the resulting output, we selected species with a mean prevalence exceeding 0 in all samples, excluding the *Xanthomonas_perforans_55843*, instead the pangenomes of identified species id (mentioned according to MIDAS default database: (*Pseudomonas_oleovorans_57108, Methylobacterium_sp_59341, Pseudomonas_fulva_57974, Brevundimonas_nasdae_60942, Methylobacterium_radiotolerans_54853, Alpha_proteobacterium_59626, Methylobacterium_sp_58573, Methylobacterium_populi_61518, Sphingomonas_phyllosphaerae_58541, Sphingomonas_sp_60678, Sphingomonas_taxi_60871, Sphingomonas_sp_61953, Pantoea_agglomerans_54643, Pantoea_vagans_57743, Methylobacterium_oryzae_55240, Pseudomonas_fulva_58092, Methylobacterium_mesophilicum_62490, Pantoea_sp_60701, Microbacterium_paraoxydans_56209, Pseudomonas_sp_59673, Xanthomonas_arboricola_57436, Sphingomonas_sp_58049, Xanthomonas_axonopodis_56719, Methylobacterium_extorquens_57587, Xanthomonas_axonopodis_61257, Aureimonas_ureilytica_58716, Xanthomonas_axonopodis_57683, Pseudomonas_rhizosphaerae_61010, Pantoea_agglomerans_56951, Pseudomonas_fluorescens_61150, Leclercia_adecarboxylata_62497, Enterobacter_cloacae_58148, Cronobacter_zurichensis_60329, Stenotrophomonas_maltophilia_62375, Streptomyces_sp_60263, Streptomyces_sp_58511,* and *Rhodanobacter_sp_59306*) were concatenated and subjected to BLAST (blast+) against a customed MIDAS database of non-redundant *Xp* strains pangenome. Subsequently, we extracted

gene sequences with a similarity of >= 97% from the database and removed these genes from all the sample reads using bbduk (v37.36) (Bushnell 2014). The number of removed reads due to blacklisted genes is documented in the table (Supplementary Table).

Estimation of gene changes and Minor allele frequency in pathogen population

For estimating gene changes in the pathogen population, we employed MIDAS (version 1.3.2) (Nayfach, 2015/2022), processing paired-end reads in fastq format via "run_midas.py genes." We used a custom database with default settings, designating a copy number of 0.35 as the threshold for classifying a gene as absent in the sample. The above-mentioned identified blacklisted genes, exhibiting more than 97% identity, were discarded from the output files using RStudio. Gene annotations were performed using EGGNOG-MAPPER (Cantalapiedra et al., 2021) and NCBI blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). To calculate the minor allele frequency, we ran "midas.py snps" and merged the resulting output files using the default settings for average read depth and reference coverage. Subsequently, we removed SNV sites associated with blacklisted genes in addition to those with less than 10 reads depth across all samples using RStudio. We removed the SNV sites belonging to the blacklisted genes in our output files. Mummer (v3.0) (Kurtz et al. 2004; “MUMmer” n.d.) was used to identify the genome of particular minor alleles in the population.

Within-host nucleotide diversity, Mean Mutation rates per site, and selective pressures

We employed the samples (without Blacklisted genes) in our subsequent analysis to assess diversity. To gauge within-host nucleotide diversity (π), Tajima's D, and the ratio of non-

synonymous to synonymous polymorphisms (pN/pS), we utilized MetaPop (Gregory et al., 2022). For these estimations, we employed MetaPop's local alignment algorithm, which normalizes diversity estimates by dividing them by the genome length to account for uneven coverage across all samples. Also, it excludes SNP positions not covered in the genome length and sets a PHRED score threshold of >= 20 for local SNP calls (Gregory et al., 2022). Our input files for MetaPop consisted of sorted BAM files, obtained by mapping the samples (without Blacklisted genes) to the non-redundant pangenome of *Xp* strains AL22 and AL65, serving as the reference genome. We performed this mapping using BWA-MEM v0.7.12) (H. Li & Durbin, 2009), followed by the removal of low-quality alignments and duplicate reads using samtools (v1.11) (Li et al., 2009) and picard (v1.79) (https://broadinstitute.github.io/picard/), respectively. To identify genes under positive selection, we refined the output by excluding genes with a pN/pS (non-synonmous to synonymous polymorphism) ratio of less than one and a Tajima's D value greater than zero. Additionally, we computed the population mutation rate per site, θ (based on Watterson's estimate), for each treatment using Rhometa (v1.0.2) (Krishnan et al., 2022). The program uses aligned BAM files (mentioned above) and the reference fasta file (the non-redundant pangenome of *Xp* strains AL22 and AL65) as input to run freebayes to identify variant sites. Rhometa uses dataset depth to calculate *q* estimate instead of number of genomes as typically implemented in LDhat since exact number of genomes is unknown in metagenome samples. For statistics, Shapiro test was run at first to check the distribution of our dataset. Then, the Kruskal-wallis rank sum test and Dunn test were performed for overall and pairwise comparisons, respectively, for within-host nucleotide diversity, mean mutation rates per site, and selective pressure (tajima'sD and pN/pS ratios) and plotted using the ggplot2 library in R.

Pairwise host comparisons and CMH test

POPOOLATION2 software package was used to calculate the pairwise Fst using the parameters: --min-count 2 --min-coverage 4 --max-coverage 120. Cochran–Mantel–Haenszel test statistics (CMH test) implemented in the POPOOLATON2 software was used to identify the consistent SNPs among all biological replicates with the parameters settings: --min-count 2 --min-coverage 4 --max-coverage 120. SNPs with p-values above the Bonferroni-corrected significance threshold was considered as outliers. Genome-wide SNPs were plotted in the Manhattan plot using ggplot2 in R. Here, we have used the blk samples for estimates.

Discussion

Numerous research studies have explored microbial diversity, their interactions, and functional responses in various spatial and temporal contexts, such as those related to either the host or climate. However, there is a limited number of studies currently investigating how microbes and plant pathogens respond to both host interactions and climate change simultaneously, especially in real-time conditions. To investigate this, we employed open-top chambers to replicate natural environmental conditions and create elevated ozone levels. We conducted our experiments using both resistant and susceptible cultivars of pepper plants, which were exposed to elevated ozone conditions throughout a growing season. For our research, we utilized two closely related *Xanthomonas perforans* strains, AL22 and AL65, which cause Bacterial leaf spot disease in peppers and tomatoes. These strains were recovered from the resistant and susceptible pepper cultivars in Alabama, aiming to mimic the natural environment where pathogen populations consist of multiple strains. We adopted a metagenomics approach to gain insights into the evolutionary trajectories of these pathogen strains.

Our initial observation revealed a significant and fluctuating disease severity within the pathogen population. Notably, the disease severity in the resistant cultivar exposed to elevated ozone conditions suggested the potential erosion of quantitative resistance. Intriguingly, there was no significant difference in the pathogen population, but it displayed a high level of variability, which could be indicative of the pathogen's plastic response to changing climatic conditions. The increased disease severity can be attributed to several factors. First, alterations in the host's defense mechanisms may play a role. Research has indicated that ozone exposure can harm plants by generating excessive reactive oxygen species, overwhelming the plant's inherent defense mechanisms. This oxidative stress occurs when O_3 enters the plants through stomata and disrupts various physiological processes, including photosynthesis and resource allocation. Moreover, the changes induced by O_3 exposure in gene expression and protein accumulation overlap with the responses observed in plants defending against pathogens (Grulke and Heath 2020; Y. K. Sharma and Davis 1997; Tiwari and Agrawal 2018). The third factor, as previously explored by Bhandari et al., 2022., relates to the response of microbial communities to modifications not only in their composition but also in the interactions among their members and the compromised functions of the community in the face of host defense and changing environmental conditions. In our study, our primary focus was to investigate alterations in pathogen efficiency, tracking the pathogen's evolution in real-time conditions.

Our initial observation of a plastic response prompted us to investigate strain-strain interactions, and we noted a preference for the colonization success of AL65. This preference was linked to higher disease severity, as evidenced on both susceptible and resistant plants exposed to elevated ozone (see Figure 5-1). It is possible that AL65 is capitalizing on the suppression of host immunity by AL22 on the resistant cultivar, which may explain the coexistence of both strains on

the resistant plants. This strategy of maintaining a heterogeneous population could be a response to dealing with host resistance and abiotic stress. Our results indicate that it was not the environment alone that influenced the structure of the pathogen population. Instead, it was the interaction between the host genotype and the environment that led to increased susceptibility, thus altering the dynamics among the strains. The higher disease severity values observed under elevated ozone on resistant cultivars and the increased variability cannot be solely explained by the absolute abundance of *Xanthomonas*. However, we do observe an association with modified strain dynamics, which may suggest a changed ecological niche under elevated ozone conditions in the resistant cultivar. Our findings align with several studies demonstrating that microorganisms can enhance their chances of surviving challenging conditions by increasing diversity within their populations or genetic variations (Aertsen and Michiels 2005; Balaban et al. 2004; Hiramatsu et al. 2001; Lidstrom and Konopka 2010; Longo and Hasty 2006).

The variations in nucleotide diversity on the resistant cultivar may signify the phenotypic plasticity of the pathogen population, a response to the selection pressures imposed by host defenses or altered ozone levels. Notably, nucleotide diversity values remained stable and unvaried on susceptible cultivars under elevated ozone conditions, indicating that elevated ozone alone was not the primary driver of genetic differentiation in the pathogen population. This pathogen's adaptability and responsiveness to environmental fluctuations can lead to phenotypic diversity and genetic differentiation, as documented in various biological systems such as intestinal microbiota, coral reefs, marine life, wildlife populations, and microbiome interactions (Candela et al., 2012; Juan A. Bonachela et al., 2022; Leray et al., 2021; Reusch, 2014; Risely et al., 2023; Torda et al., 2017). In addition, the examination of mutation rates in pathogens originating from susceptible cultivars revealed a consistent rate of approximately 8 x 10^-5 per site, regardless of the

environmental conditions. However, in response to host resistance and altered environmental conditions, the mutation rate increased to approximately 4.2 x 10^-4 per site. Spontaneous mutations generally occur at a frequency ranging from 1 in 10^5 to 10^8 and introduce random genetic variations within populations. Pathogen populations can quickly showcase significant phenotypic diversity through the introduction of point mutations (Drake 1991; Garibyan et al. 2003; Lee et al. 2012).

The co-evolved host-pathogen interaction indicates the selection pressure towards the pathogen population, which would reflect variants in the genetic architecture of the pathogen genome (Duxbury et al. 2019). Stress-induced mutagenesis theory states microbial populations have higher rates of genome-wide mutation/ variations including gene gain and loss events, recombination, insertion, and duplications, facilitating the evolution by increasing the plasticity pathogen evolution by increasing the plasticity (Albalat & Cañestro, 2016; Chu et al., 2021; Zhang et al., 2016). In this context, it becomes evident that the mere presence of strain heterogeneity does not adequately account for the increased levels of genetic differentiation observed across different environments, particularly in the case of resistant cultivars. Rather, it is likely that evolutionary adaptations have played a role in generating higher levels of nucleotide diversity within the host under elevated ozone conditions compared to ambient conditions (see Figure 5-2).

The evolutionary modifications were quantified in which independent novel mutations sweep to high frequency from mid to end of season. It was seeded with a mixture of parallel in both de novo mutations (4 novel alleles) and rapid mutations among different replicates in response to environmental change and host resistance (Albalat and Cañestro 2016). The 4 novel parallel alleles: one belongs to an unknown gene and the other three alleles to intergenic regions (IGRs), one IGR allele are linked to genes related to flagellar type III secretion system proteins and the

other two IGRs are connected with genes of domain-containing protein and bifunctional biotin--[acetyl-CoA-carboxylase] ligase/biotin operon repressor BirA. We observed the alleles swept to high frequency from lower by the end season might have allowed pathogen to adapt rapidly using those mutations in response to host resistance and elevated ozone conditions. Those alleles were related to Acid phosphatase genes, which could have provided the ability to the pathogen population to resist oxidative stress of reactive oxygen species produced by plants upon invasion or elevated ozone. (Bhadouria and Giri 2022; Jungnitz et al. 1998). However, further studies are required to confirm the functional significance of these changes in the fitness and virulence of the pathogen population.

We observed the strong purifying selection in the pathogen population irrespective of cultivar and conditions, which acts against newly emerged harmful mutations, preserves the genetic traits, and leaves imprints on genetic diversity by alteration in the distribution of genetic variants at specific sites (positive selection figure) (Cvijović, Good, and Desai 2018). The high and variable within-host polymorphism types (Figure 5-2.F) in the population from resistant cultivars under both conditions during mid-season and reduction during the end indicate filtering of harmful mutations in the population. Further, the accumulation of common significant SNPs in the pathogen populations emphasizes the independent evolution of crucial traits parallelly across pathogen populations from susceptible cultivars and resistant cultivars under both conditions. The common significnrt SNPs were common across pathogen population from resistant and susceptible cultivar in elevated ozone, were Intergenic region, putative protein and molybedunum cofactor guanylyltransferase (MobA).

The horizontal/lateral gene transfers and natural selection can drive the gene acquisition and loss process in the evolutionary process of microbial populations. Variations in the rate of gene gain and loss have impacts on the pathogen's fitness which contributes towards overall diversity of the pangenome (Brockhurst et al. 2019; Domingo-Sananes and McInerney 2021; Lefébure and Stanhope 2007; Moulana et al. 2020). The pathogen adapted with the strategy of "less is more" under the host defense and elevated ozone conditions that led to more gene loss events because of redundancy in functions can have fitness effects on overall populations (N. Li et al. 2017; Seidl and Thomma 2017; Simonsen 2022). Overall, the observed gene gain loss events from different strains are indicative of the dominance of respective strains under different conditions. However, COG categories [C] (energy production and conversion), [F] (nucleotide transport and metabolism), [P] (inorganic ion transport and metabolism), [L] (DNA replication, recombination, and repair), [E] (amino acid transport and metabolism) and [K] (transcription), would be important for elevated because of its gain in pathogen under concurrent stress and elevated ozone stress (Figure 6), which are consistent with upregulation of genes in prokaryotes under stress. Genes related to [K] and [L] are more conserved than nonessential genes in bacteria subject to strong selective pressure (Luo, Gao, and Lin 2015). When DNA damage occurs under stress, SOS response gets activated, which results in DNA repair or recombination. It can increase the genetic diversity and mutation rates in the populations (Foster 2005). Cells that lack or have modified the necessary proteins for repairing double-strand breaks (DSBs) in DNA exhibit distinct disruptions in genome replication and the overall viability of the cells (Sinha, Possoz, and Leach 2020). The various alterations in the environment (high humidity, soil drought, and increasing ozone) have resulted in metabolic adjustments and shifts in the balance of carbon and nutrients, that can indirectly affect the osmotic or salinity property of plants (Oksanen 2021). That is why

we observed a high influx of genes related to COG [P]. However, the functional validation of these COG category genes was not studied for virulence, which is a limitation of this study.

Our study has a few limitations, including a relatively small number of sampling points and a lack of long-term data due to not preserving samples from the population or conducting tests over multiple years. In future work, it is essential to investigate the functional significance of the observed mutations, explore unknown genes that might play a significant role, assess the fitness of the pathogen population under varying environmental conditions, and integrate our findings with data on other climatic factors for a more comprehensive understanding. Further research should also delve into the host's role and responses in these dynamics, among other relevant factors. Despite these limitations, the data we've gathered remains valuable and can be used for modeling approaches to predict pathogen evolution, contributing to our ability to anticipate and manage pathogen dynamics effectively. Overall, we observed the rapid evolution of the pathogen population on resistant cultivar during a single growing season to altered environment conditions. It is an alarming situation for the durability of resistant varieties under future climatic conditions, we need to focus on more breeding strategies against pathogen and altered environment at the same time.

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