Mixed Infection in Tomato Plants by Dominant Bacterial Spot Pathogen and the Co-occurring Bacterial Species

by

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

Auburn, Alabama
May 7, 2022

Keywords: *Xanthomonas*, *Pseudomonas*, tomato coinfection, mixed infection, bacterial leaf spot disease

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Abstract

Pathogen dynamics can be altered by co-occurring pathogenic or opportunistic entities inhabiting the same host species. Mixed infection or co-infection caused by multiple pathogenic strains are common in nature. However, traditional plant pathogen studies have always focused on the binary interaction between single host and single pathogen. In this study, I have looked beyond the binary interaction between single host and single pathogen and observed the effect of co-occurring bacterial species on the pathogenesis of dominant pathogen. *Xanthomonas arboricola* and various *Pseudomonas* species co-occur with the bacterial leaf spot pathogen *Xanthomonas perforans*. Altered disease severity was observed when tomato plants were co-inoculated with these three bacterial species. In-planta population of each of these bacterial species revealed that the dominant pathogen *X. perforans* has the highest population when it was present alone, but its population decreases in presence of the co-occurring bacterial species. Presence of *X. perforans* always induced the in-planta population of *X. arboricola* and the *Pseudomonas* species. PAMP triggered immunity (PTI) assay revealed the inability of the *Pseudomonas* sp. to suppress PTI. Genome analysis confirmed the lack of effector molecules in the *Pseudomonas* species. The principal cell wall degrading enzymes were not detected in the *Pseudomonas* species. Similar nutritional profile of the three bacterial species indicated niche overlap and potential competition for resources which explained the decrease in *X. perforans* population. In mixed infection the *Pseudomonas* species and the *X. arboricola* were observed to colonize the apoplast in the later stage of the infection after *X. perforans* already colonized it. Our study revealed that mixed infection with the weak taxa changed the population dynamics of the dominant taxa whereas the weak bacterial species could exploit the dominant pathogen to colonize the host plant.

Acknowledgements

I would like to express my sincere gratitude to my major advisor, Dr. Neha Potnis for her patience and constant support. I would also like to express my deepest appreciation to my committee members Dr. Leonardo De La Fuente, Dr. Kathy Lawrence, and Dr. Courtney Leisner for their lenience and support throughout my course of study. I also thank Dr. Bernardo Chaves-Cordoba for helping me with data analysis.

I would also like to thank my lab members, Rishi Bhandari, Prabha Liyanapathiranage, Destiny Brokaw and Michelle Pena for their support, suggestions, and encouragement. I am extremely grateful to Auston Holland for providing me with the plants used for my experiments.

Finally, I especially thank my family for their love and support.

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1. CHAPTER ONE

Introduction & Literature Review

Introduction

Microbes in nature exist as a part of complex multispecies community (Bengtsson-Palme, 2020). The interactions among multiple species in a bacterial community is much more diverse and as one single community they can perform actions which help them to survive better (Bauer et al., 2018). Compared to the majority of host associated microbiomes, bacterial communities inhabiting the leaf phyllosphere have a shorter duration of survival on the host (Koskella, 2020). In the past, bacteria were considered to lack the ability to interact, but this view has changed and now we know that they can communicate and organize into groups, which are important parameters that help them with virulence (Greenberg, 2003). There can be co-operative or competitive interactions among plant microbiota members. Two or more bacterial species can have nutritional interdependencies and co-operate with each other. This type of interaction allows microbes to extend their fundamental niches, get access to compounds that are not easily available and survive in nutrient-poor environments (Harcombe, 2010). Nutritional interdependencies usually support beneficial interactions among community members (Morris et al., 2013). Nutritional resources can also be a focal point of microbial competition (Hibbing et al., 2010). Scientists have discovered that bacteria mainly inhibit the growth of metabolically similar species (Russel et al., 2017). It has also been recorded that plant-associated bacteria can engage in direct antagonistic interactions mediated by contact-dependent killing mechanisms. Contact dependent competition is usually mediated by bacterial type VI secretion system, which can inject toxins or effector molecules into opponent cells (Records, 2011). Various plant-associated bacteria can secrete chemical

compounds, metabolites that have the ability to directly repress the growth of their opponents (Raaijmakers et al., 2002; Raaijmakers & Mazzola, 2012). In some cases, populations of micro-organisms consume metabolites released by other microbial taxa, which is referred as facilitation. Waste products produced by one microbial species can be consumed by another one with complementary metabolic capability. Consumption of waste products is referred as syntrophy (Douglas, 2020). In some bacterial interactions, one population benefits and the other population is unaffected. Such interactions are referred as commensalism and are common in biofilm systems (James et al., 1995). Interactions among microbes might alter plant growth and fitness. However, these interactions might not always influence the plant health in a positive way, but it can also be detrimental for the plant by enhancing disease (Hassani et al., 2018). To understand the potential interactions among the various microbial species that inhabit the host, studying the co-occurrence pattern can be used as an important tool (King et al., 2012). The relationship between co-occurring bacterial species is considered ecologically important because it can also provide valuable information on host-microbe interaction (Williams et al., 2014). However, co-occurrence should not be considered as an evidence of ecological interactions because it remains unclear to what extent the signal of interaction can be captured from co-occurrence observational data (Blanchet et al., 2020).

Most disease studies ignore co-occurring pathogens including weaker or opportunistic pathogens, and focus on one single dominant pathogen, and thus in turn do not consider possible inter-species interaction between microbial pathogens. Researchers have observed that mixed infection between more than one microbial pathogenic species can develop complex plant disease (Lamichhane & Venturi, 2015). Increased disease severity because of synergistic microbial interaction has also been observed in both humans and animals (Abdullah et al., 2017).

Coinfection or Mixed infection

Coinfection or mixed infection is the simultaneous infection of a single host plant (or in a single lesion) by multiple pathogen species. In agriculture, various diseases of economically important crops are caused by mixed infection or coinfection of more than one pathogenic agent, who are comparatively less virulent in single infections. Disease outcome of co-infection depend on the extent to which each of the pathogen colonizes the host. It also depends on timing of colonization (Benítez et al., 2013). Single inoculation time points have been used for most of the coinfection studies. Therefore, the possible influence of timing was not clearly understood. In natural or field conditions sequential coinfections are more likely to be present rather than simultaneous coinfection. Studies reveal that sequential and simultaneous coinfections led to differences in disease severity (Marchetto & Power, 2017). Development of severe disease symptom has been observed in multiple studies where hosts had mixed infection with multiple microbial species (Lamichhane & Venturi, 2015). In 2008, a study done in the Czech Republic showed mixed infection caused by *Pseudomonas corrugata* and *Pseudomonas marginalis* caused a sudden collapse of tomato plants with severe disease symptoms (Kůdela et al., 2010). Another study done in 2013 shows increased disease severity in young grapevines caused by co-inoculation of *Ilyonectria macrodidyma* and *Botryosphaeriaceae* isolates (*Diplodia seriata* or *Diplodia mutila*) in both field and lab conditions. Growth and fruit yield were significantly diminished in the co-infected plants of vineyard (Whitelaw-Weckert et al., 2013).

Increased disease severity and reduced plant growth was observed when alfalfa plants were co-inoculated with *Fusarium oxysporum* f. sp. *medicaginis* and *Rhizoctonia solani* (Fang et al., 2021). Multiple infections are inevitable if a host is exposed to a pathogen for long enough, because the

pathogen can induce physiological changes which might alter the infected host's vulnerability towards the following infection (van Baalen & Sabelis, 1995). Under multiple infection the highest virulence of an infectious agent is often different from that under conditions of single infection. This difference or change depends on the nature of the interaction between the different parasite genotypes within the same host. The interaction can be competitive or cooperative (López-Villavicencio et al., 2011). In species rich plant communities, host plants are less susceptible to get infected with multiple pathogen or coinfection (Rottstock et al., 2014). Variation in the pathogen aggressiveness influences the probability of the pathogens to successfully coinfect the same plant host, which indicates that pathogen traits play a crucial role in the progress of coinfections (Susi & Laine, 2017). It is expected that co-infection will always have worse effects on the host than infection with a single pathogen. However, this might not always be true (Stubbendieck & Straight, 2016).

Interaction between bacterial pathogens in Co-infection or Mixed infection

Both plants and animals are often coinfected with pathogen species. Interactions between co-infecting pathogens are usually detected by changes in the density of the dominant pathogen, because of the presence of another confecting species. Limited resources, host immune responses or direct interference like release of toxins are some of the few reasons why two or more cooccurring species or pathogens interact. While fighting for resources, some pathogen might have a better nutrition acquisition capability if they can secrete cell wall degrading enzymes or toxins to sequester host nutrients (Abdullah et al., 2017). When bacterial competitors are competing for resources, the competition can be exploitative and indirect. The competitors can also directly harm

each other resulting in cell damage. This method of competition is referred as interference competition (Cornforth & Foster, 2013).

Interference competition among co-infecting pathogens can occur by production of bacteriocin or any other harmful molecules. Another type of competition may exist between co-infecting pathogen species, where the increasing density of one of the co-infecting pathogens triggers the host immune response, which creates adverse condition for all the co-infecting pathogen. This type of interaction is referred to as apparent competition (Kinnula et al., 2017a; Read, 2001). Presence of a competitor influences the bacterial species to expend more in biofilm formation which causes phenotypic changes and gives more protection from a hostile environment. In a mixed infection, bacterial populations can cooperate by sharing virulence factors, siderophores, toxins, exo-enzymes, and bio-surfactants. For examples, iron is a limited resource in the host environment. Competition for iron acquisition can be considered as a reason for bacterial species to compete in the host environment. Interaction between different bacterial species influences their lifestyle and how they communicate. Inter-species quorum sensing is a possible mode for communication between different bacterial species who are in proximity (Rezzoagli et al., 2020). Opportunistic pathogens and other co-occurring microbes might exploit these characteristics in mixed infections. In all mixed infections, microbes compete for host resources, and competition selects for a higher level of virulence. Virulence is proportional to a higher density of the pathogen. In mixed infection models, the only virulence reducing mechanism proposed is different forms of cooperation (Choisy & de Roode, 2010). Relatedness among the co-infecting pathogen strains plays a key role in the virulence of mixed infection and the pattern of interaction between the co-infecting pathogens (Buckling & Brockhurst, 2008; Kinnula et al., 2017b; Tollenaere et al., 2016).

Role of plant host immune response in mixed infection

When a pathogen enters a host plant, it does not only encounter co-occurring microbial species but also the host's defense or immune response. The ability of a pathogen to colonize and grow on its host depends on the diverse microbiome of the host. Successful colonization or infection by a plant pathogen depends on the presence of resistance gene in the host plant. When a corresponding plant disease resistance *R* gene, recognizes the a virulence *avr* gene, it results in disease resistance (Dangl & Jones, 2001). Interaction between a commensal and a host plant is usually balanced and asymptomatic. However, if a virulent pathogen has already compromised the host's defense mechanism and it rendered it susceptible, then a bacterial species which is otherwise avirulent might also be able to colonize the host. Changes in metabolism can be induced by pathogen signals which can alter the host's defense response (Abdullah et al., 2017; Chisholm et al., 2006; Dodds & Rathjen, 2010).

In this study, influence of coinfection by *Xanthomonas* and *Pseudomonas* on overall disease severity and dominant pathogen population dynamics on tomato was investigated. It is not known if mixed infection can change the bacterial leaf spot disease progress in tomato. Studying mixed infection caused by *Xanthomonas perforans* and its co-occurring bacterial species will give us a better perspective in understanding of the behavior of the *Xanthomonas perforans* population during coinfection. It will also help us learn more about the pathogenesis strategy of *X. perforans* This knowledge can be utilized in the future to control bacterial spot of tomato. The effect of humidity on co-infection by bacterial leaf spot pathogen and co-occurring taxa has also been investigated in this study to understand the effect of environmental conditions on the disease development.

Bacterial spot of tomato

Tomato is one of the major commodities of the southeastern United States. Various diseases play a major role in reducing the quantity and quality of tomato. Among the diseases caused by bacteria in tomato, bacterial spot disease is the most important one. Bacterial spot disease of tomato can be devastating and lead to significant yield loss (Sharma & Bhattarai, 2019). In the southeastern United States, *Xanthomonas perforans* is the dominant pathogen that causes bacterial spot disease of tomato, and it is a common disease in transplant houses and fields (Abrahamian et al., n.d.).

The bacterial leaf spot pathogen *Xanthomonas* belongs to the class Gamma-proteobacteria. It's a rod-shaped gram-negative bacterium with a polar flagellum. Bacterial leaf spot of tomato is caused by four different species of *Xanthomonas*. *X. perforans*, *X. euvesicatori*a, *X. gardneri* and *X. vesicatoria.* In the southeastern United States *Xanthomonas perforans* is the dominant pathogen that causes bacterial spot of tomato (Abrahamian et al., 2021). The pathogen produces a yellow pigment, xanthomonadin. Type III secretion system (T3SS) and associated effectors play a key role in causing the pathogenicity by *Xanthomonas perforans*.

Symptoms, and identification

Bacterial spot disease of tomato prevails worldwide. Initial symptoms are appearance of small, water-soaking lesions. As the disease progresses, they develop into small brownish black spots. In humid weather, spots may emerge and produce larger necrotic spots. Leaf tissue might turn brown and die if leaf spots are abundant. Lesions caused by *Xanthomonas perforans* can mature into shot holes. The causal pathogen or bacteria can be identified by streaking the infected leaf tissue on

nutrient agar media. Production of yellow pigments (xanthomonadins) in mucoidal colonies indicate the presence of *Xanthomonas* spp. in the diseased leaf tissue (Potnis et al., 2015).

Co-occurring microbial species in the tomato phyllosphere

Co-occurring microbes can influence the fitness of the dominant pathogen (Newberry et al., 2020). However, it has been studied that only a weak relationship exists between co-occurrence and interactions. Therefore, co-occurrence should not be considered as an evidence of ecological interaction (Blanchet et al., 2020). Microbial community analysis of the tomato leaf phyllosphere revealed that co-occurrence of *Pseudomonas* species and *Xanthomonas* spp. is common in Alabama fields (Newberry et al., 2020). Pectolytic, opportunistic, avirulent *Xanthomonas* species have also been reported to be isolated from mixed infections with *Pseudomonas syringae* from pepper and tomato transplants (R. Gitaitis et al., 1987). Surveys carried out in soil-grown tomatoes in Sicily (Italy) showed that *Pseudomonas* spp. strains were frequently associated with *Xanthomonas perforans*, causal agent of tomato pith necrosis. Six strains belonging to *Pseudomonas fluorescens*, *P. putida*, *P. citronellolis* and *P. protegens* species significantly increased pith necrosis and vascular discoloration symptoms when co-inoculated with *X. perforans* on tomato plants. In these co-inoculations, *X. perforans* population density was significantly higher than that of *X. perforans* inoculated individually (Aiello et al., 2017). Reports of weak Xanthomonads being isolated in transplant houses, and the association of *Pseudomonas* species particularly *Pseudomonas cichorii* with *Xanthomonas* species has also been recorded.

Pseudomonas species as a pathogen

Among the *Pseudomonas* species, *Pseudomonas syringae* has been studied extensively to understand the mechanism of plant-microbe interaction. Pathogenic *Pseudomonas* outbreaks are aided by cool weather and rainfall. However, species like *Pseudomonas cichorii* is considered as warm weather pathogen and they can also grow under humid conditions (Timilsina et al., 2017). Different pathovars of *P. syringae* have been identified. They can infect various economically important crops. *P. syringae* has both an epiphytic and endophytic phase. Some strains are strong, and some are weak epiphytes. They can cause disease during the endophytic phase while multiplying in the apoplast. Their ice nucleation ability facilitates bacterial entry by causing injury or creating openings on the leaf surface. Multilocus sequence analysis of *P. syringae* species complex has divided it to 13 phylogroups. *Pseudomonas cichorii* is one of the phylogroups that belong to the *P. syringae* species complex. They have the single part pathogenicity island (S-PAI). Single-part pathogenicity island (S-PAI) encodes AvrE and/or HopM1 effectors which are associated with apoplast water soaking. S-PAIs do not have a conserved effector locus (encodes conserved effector genes like hopAA1-1, hopM1 and *avrE*) but conserved effectors of *hrp-hrc* cluster, which encodes type 3 secretion system (T3SS), can be present. T3SS facilitates the delivery of bacterial effector proteins into the host cells which will govern the disease development or pathogenesis. *Pseudomonas cichorii* do not carry the ice nucleation gene (INA). *Pseudomonas cichorii* is one of the early branching lineages of *P. syringae* species complex and they have a broad host range (Xin et al., 2018). *Pseudomonas cichorii* can cause browning symptoms and bacterial rot on economically important plants like lettuce. The symptoms caused by *P. cichorii* on lettuce are similar to soft rot. However, *P. cichorii* does not produce the principal cell-wall degrading enzyme pectate lyase which indicates that *P. cichorii* uses a different mechanism to

cause the rot. *P. cichorii* has been observed to cause plant cell death by inducing apoptotic cell death as a part of their pathogenesis. Disease development by *Pseudomonas cichorii* in lettuce depends on protein synthesis of lettuce cells. Lettuce leaf tissue collapse by *P. cichorii* is suppressed by the concomitant presence of Cycloheximide, a protein synthesis inhibitor specific for eukaryotes. The comprehensive role of plant metabolism, which renders the plant susceptible to disease development is still unknown. Microscopic observation of *P. cichorii* showed that they can colonize the intercellular spaces of the epidermis and mesophyll and then moved into the vascular bundle. The disease develops following the bacterial growth and movement in a lettuce leaf (Hikichi et al., 1998). Whole genome sequencing of *Pseudomonas cichorii* JBC1 strain has revealed that JBC1 strain encodes for type IV pili, the cell wall anchored protein SasA, and outer membrane fibronectin-binding protein which play an important role in attachment of the pathogen to the host plant. It also codes for the phytotoxin coronatine (causes stomata to re-open after the stomata close in response to PAMP or pathogen associated molecular pattern). JBC1 encodes 200 different genes for membrane transport mechanisms, such as ABC transporters and components of type I, II, III, IV, and V secretion systems. These components are essential for multiplication and virulence of the pathogen in the host plants. Cell wall degrading enzymes. Pectinlyase and polygalacturonase, the two chief cell wall degrading enzyme encoding genes were not found in the JBC1 genome (Ramkumar et al., 2015). *P. cichorii* has been reported as highly virulent and the type of the host plant regulates the expression of this pathogen's virulence mechanism (Hikichi et al., 2013).

Patho-system and Objective of the Study

Environmental conditions like weather and locations influence the composition of the leaf microbiome (Leveau, 2019). The microbiota can alter various physiological processes of plants under different conditions. Therefore, the plant microbiome is considered as a key determinant of plant health. Studying the phyllosphere associated microbiome of plants and how they interact, can lead us to strategies to practice sustainable agriculture like developing microbial inoculants for plant health or designing biocontrol agents to fight pathogens (Parasuraman et al., 2019).

In a metagenomic study done by Newberry et al., 2020, co-occurrence patterns of bacterial species with respect to tomato bacterial leaf spot pathogen, in the tomato fields of southeastern United States was analyzed. This study revealed presence of *Xanthomonas* species and *Pseudomonas* species along with the tomato bacterial leaf spot pathogen (Newberry et al., 2020). It was also discovered that a single lesion can have simultaneous presence of the dominant pathogen *X. perforans* and *X. arboricola*. Another study accounted for the presence of pectolytic *Xanthomonas* and *Pseudomonas* species in mixed infections with a dominant plant pathogen *Xanthomonas campestris* pv. *vesicatoria* (R. D. Gitaitis, 1987). Interaction between tomato pith necrosis causing *Xanthomonas perforans* and different species of *Pseudomonas* has also been observed in surveys carried out in soil grown tomatoes in Sicily (Aiello et al., 2017).

Our aim was to investigate the outcome of co-infection caused by co-occurring bacterial species. Therefore, to study the interaction among co-occurring bacterial species and the dominant bacterial leaf spot pathogen we chose *Xanthomonas arboricola* and *Pseudomonas* species as two candidates, which co-occur with *X. perforans* in the tomato leaf phyllosphere in southeastern United States.

Initial co-inoculation experiments with the three bacterial species were done to investigate if there were any differences between disease severity of tomato plants inoculated with only *Xanthomonas*

perforans and mix-inoculated with *Pseudomonas* species, *Xanthomonas arboricola* and *Xanthomonas perforans*. Increased disease severity was observed in tomato plants co-inoculated with the three bacterial species which led us to move forward and investigate further to understand the reason behind altered disease severity using the following objectives, which has been elaborately discussed in the next two chapters.

1. Studying the influence of weak/opportunistic bacterial species on bacterial leaf spot pathogen (*Xanthomonas perforans*) population dynamics in planta.
2. Dissecting the interactions among the three taxa using in vitro experiments and genome analysis.

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2. CHAPTER TWO

Influence of weak co-occurring bacterial species on dominant tomato bacterial spot pathogen population dynamics in planta

Introduction

Tomato is one of the major commodities of the southeastern United States. Various diseases play a major role in reducing the quantity and quality of this crop. Bacterial spot disease is one of the yield-limiting endemic diseases in the southeastern U.S. (Horvath et al., 2012; Sharma & Bhattarai, 2019). *Xanthomonas perforans* is the dominant pathogen that causes bacterial spot disease of tomato (Liao et al., 2019). It is a seedborne disease and transplant houses are considered as a major source of outbreaks (Abrahamian et al., 2019).

While most of the studies have highlighted the importance of the dominant *Xanthomonas perforans* pathogen on tomato, a handful of studies have noted the occurrence of mixed infections with other weak or opportunistic pathogens in transplant houses or field conditions. In a 1987 survey, avirulent, pectolytic and opportunistic xanthomonads were isolated along with other dominant xanthomonads and pathogens like *Pseudomonas syringae* from tomato transplants with foliar spots caused by mixed infection. None of the avirulent xanthomonads produced typical bacterial spot symptoms on susceptible plants but these strains can be considered as opportunistic pathogen, given their pectolytic ability (Gitaitis et al., 1987). These avirulent xanthomonads later were identified as *Xanthomonas arboricola* (Vauterin et al., 1995). Similar reports of association of *Xanthomonas arboricola* with bacterial spot infected tomato and pepper have been noted in the subsequent years (Mbega et al., 2012, Myung et al., 2010, Newberry et al. 2020).

Another co-occurring genus in bacterial spot infected fields observed in the recent outbreaks is *Pseudomonas* (E. Newberry et al., 2020). In 2017, a survey carried out in Italy showed that

Pseudomonas spp. strains were frequently associated with *Xanthomonas perforans*, causal agent of tomato pith necrosis. Six strains belonging to *Pseudomonas* species significantly increased pith necrosis and vascular discoloration symptoms when co-inoculated with *X. perforans* on tomato plants. *X. perforans* populations in these co-inoculations were significantly higher than that of *X. perforans* inoculated individually (Aiello et al., 2017).

Besides studying the binary relationship between a single plant host and its single pathogen, there is an increasing interest in studying co-occurring bacterial species and the nonpathogenic interactions between plant tissue and bacteria (Baldotto & Olivares, 2008; Sturz et al., 2000). The relationship between co-occurring bacterial species are ecologically important because it can provide important information on microbial interaction and host-microbe interaction (Williams et al., 2014). These host-microbe or microbe-microbe interactions are not necessarily negative for the plant, and there are plenty of interactions from which the plant can benefit through direct or indirect effects of the associated microbes (Schirawski & Perlin, 2018). The co-occurring bacterial species can also coexist without harming each other. If they cause a mixed infection in the plant host, it can lead to various types of interactions among the bacterial species such as antagonism, mutualism, cooperation, or synergism (Abdullah et al., 2017). In case of plant-microbe interactions, plant defense response plays an important role, that adds a layer of complexity to the microbe-microbe interactions. Plants can recognize pathogens by two strategies: microbial elicitors like pathogen-associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs). These patterns are recognized by plant receptors proteins called pattern recognition receptors (PRRs). Stimulation of PRR induces PAMP triggered immunity (PTI) in host plants (Dodds & Rathjen, 2010). The second method of detection is recognition by intracellular receptors of pathogen virulence molecules known as effectors, which induces

effector-triggered immunity (ETI) in the host (Jh, 2015). In case of co-infection or mixed infection, the plant host's defense mechanism might prioritize to invest its defense metabolites against certain pathogens. The prioritization depends on the mode of action of the pathogen (Castrillo et al., 2017; Hacquard et al., 2016). This might make us wonder if infection by one pathogen can impact the plant's defense for the subsequent infection by a different pathogen or not (Abdullah et al., 2017). Outcomes of mixed infections caused by co-occurring bacterial species on the immune system of host plants has not been studied extensively. In this study, our aim was to understand the effect of mixed infection caused by co-occurring bacterial species on the pathogenesis and in planta population of the dominant tomato bacterial leaf spot pathogen. We also tried to investigate if plant's immune system has any influence on the dominant pathogen in case of mixed infection . *Xanthomonas perforans* and the co-occurring bacterial species *Xanthomonas arboricola* and *Pseudomonas* species were used to co-inoculate tomato plants to observe the outcome of mixed infection caused by the dominant pathogen and the co-occurring bacterial species. Higher disease severity caused by mixed infection led us to hypothesize that mixed infection caused by co-occurring bacterial species alters the population and disease dynamics of the dominant plant pathogen. To investigate our hypothesis, we have explored various in planta experiments to understand the potential role of the host plant immune system and the effect of co-occurring taxa on the pathogenicity of *Xanthomonas perforans.*

Materials and Methods

Plant material and growth conditions

Tomato plants of cultivar FL 47R, grown in greenhouse, were used for this study. After the seeds germinated, two weeks old seedlings were transplanted into 4" plastic pots with potting mix. Plants

were kept in greenhouse at 28°-30°C under greenhouse conditions for 4-5 weeks before using them for our study.

Bacterial strains, media, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 2.1 and 2.2, respectively. Bacteria were routinely cultured on nutrient agar (NA, Difco) supplemented with 15 μg/ml gentamicin or tetracycline or chloramphenicol and 50 μg/ml streptomycin where appropriate. These strains were grown for 24-48 hours at 28°C. The wild type bacterial strains *Xanthomonas perforans* AL65, *Xanthomonas arboricola* CFBP 6826, *Pseudomonas* species 93B.260 were tagged with fluorescent molecule producing gene in their chromosome according to the protocol described in Schlechter et al., 2019 (Schlechter & Remus-Emsermann, 2019). Construction of fluorescent bacterial strains was done to distinguish each strain under microscope in mixed infections. Each of the newly constructed fluorescent bacterial strain had their own emission and excitation peak and it did not overlap with one another. The sGFP2 protein expressing strain *Xanthomonas perforans* AL65::Tn7-mre152 was constructed by performing conjugation of *Xanthomonas perforans* AL65 and pMRE-Tn7-152 plasmid carrying *E. coli* S17-1 lambda pir. mTagBFP2 protein expressing strain *Xanthomonas arboricola* CFBP 6826::Tn7-mre160 was constructed by conjugation of pMRE-Tn7-160 plasmid carrying *E. coli* S17-1 and *Xanthomonas arboricola* CFBP 6826. mScarlet-I protein expressing strain *Pseudomonas* species 93B.260::Tn7-mre145 was constructed using pMRE-Tn7-145 plasmid carrying *E. coli* S17-1 and *Pseudomonas* species 93B.260. All the strains were stored in 30% glycerol at -80°C. The properties of the fluorescent proteins are listed in Table 2.3.

Table 2. 1: Bacterial strains used in this study

Strain	Short name; Features	Source
Xanthomonas perforans AL65	Xp AL65 WT, Streptomycin resistant	Laboratory collection (E. A. Newberry et al., n.d.)
Xanthomonas perforans AL65::Tn7-mre152	Xp; green fluorescent, chloramphenicol, kanamycin, and streptomycin resistant	This study
Xanthomonas arboricola CFBP 6826	Xa WT	Laboratory collection, Pena et al. unpublished
Xanthomonas arboricola CFBP 6826::Tn7-mre160	Xa; blue fluorescent, chloramphenicol, and tetracycline resistant	This study
Pseudomonas species 93B.260	Pc WT	Laboratory collection
Pseudomonas species 93B.260::Tn7-mre145	Pc; red fluorescent, chloramphenicol, and gentamicin resistant	This study

Table 2. 2: Plasmids used in this study

Name	Expressed protein	Notable features	Source
pMRE-Tn7-152	sGFP2	green fluorescent, Amp^R, Cam^R, Kan^R	(Schlechter et al., 2018)
pMRE-Tn7-160	mTagBFP2	blue fluorescent, Amp^R, Cam^R, Tet^R	(Schlechter et al., 2018)
pMRE-Tn7-145	mScarlet-I	red fluorescent, Amp^R, Cam^R, $Gent^R$	(Schlechter et al., 2018)

Amp^R, Cam^R, Tet^R, $Gent^R$, Kan^R: ^{R} indicates antibiotic resistance respectively.

Table 2. 3: Fluorescent protein properties

Name	Excitation peak	Emission peak	Emission color
sGFP2	495 nm	512 nm	Green
mTagBFP2	402 nm	457 nm	Blue
mScarlet-I	569 nm	593 nm	Red

*More detailed spectrum figure is described in Schlechter et al., 2018.

Tracking In planta populations of individual strains from mixed inoculation and disease severity measurement under high and low humidity

Bacterial cultures of overnight grown *Xanthomonas perforans* AL65::Tn7-mre152 (Xp), *Xanthomonas arboricola* CFBP 6826::Tn7-mre160 (Xa) and *Pseudomonas* species 93B.260::Tn7-mre145 (Pc) were suspended in $MgSO_4$ buffer and adjusted to OD_{600} = 0.3, i.e., to a concentration of 10^8 CFU/mL, and next the suspensions were diluted 100 times to a final concentration of 10^6 CFU/mL. In this population study 4–5-weeks-old tomato cv. FL 47R plants were inoculated by invertedly dipping for 30 seconds in 600 ml cell suspensions containing ~1 X 10^6 CFU/ml of Xp or Xa or Pc alone or Xp + Pc or Xp +Xa or Xp + Xa + Pc mixed in 1: 1 or 1: 1: 1 ratio for the mixed inoculation study. To understand the influence of humidity, each treatment were incubated under both low and high humidity. Each inoculum suspension was amended with 0.025% (vol/vol) of Silwet 77. Negative control inoculum was prepared by mixing 0.025% (vol/vol) of Silwet 77 with 600 ml of sterile $MgSO_4$. For each treatment, before inoculating the plants, the inoculum was plated to confirm the 1 X 10^6 CFU/ml concentration and 1: 1 or 1: 1: 1 ratio for the mixed inoculum. After dip inoculation, plants were kept inside closed boxes, which were placed inside growth chambers with 12h light/dark cycle and 25°C temperature. Wet paper towels were placed at the bottom part of the walls of boxes containing the plant replicates for high humidity. The closed boxes containing the plants were not disturbed for the next 48 hours to maintain high humidity. After the first two days, lids were taken off from the boxes containing plant replicates for low humidity conditions. For plant replicates with high humidity conditions, each day lids were taken off from the boxes for 12-hour light cycle and the boxes were closed back for 12-hour dark cycle. Paper towels inside the boxes under high humidity conditions were always kept wet. Leaf samples were collected two hours after the plants were dip-inoculated. Next, the

leaf samples were collected on day 0, 3, 6, 9 and 14. A sterile cork borer of radius ~ 0.5 cm was used to collect 4 circular leaf discs from each leaf. Therefore, at each sampling point ~ 3 cm^2 area of leaflet tissue was collected from each plant. A sterile forceps was used to place the leaf discs inside sterile microcentrifuge tubes containing 1 ml sterile $MgSO_4$ buffer. The leaf tissue was macerated using a sterile homogenizer (Dremel). Serial dilutions of the homogenized leaf tissue suspension were plated on selective media using spiral plater (Neu-tec Group Inc, NY, USA) to estimate the population size of Xp, Xa, and Pc in individual inoculations and mixed inoculations. Plates were incubated for 3-4 days at 28°C before enumerating the number of colonies. Bacterial populations were calculated as colony forming units (CFU) per cm^2 of leaf area. Disease severity of bacterial leaf spot was measured using the following scale and mean disease severity was calculated. Disease scale: 1 = symptomless, 2 = a few necrotic spots on a few leaflets, 3 = a few necrotic spots on many leaflets, 4 = many spots with coalescence on few leaflets, 5 = many spots with coalescence on many leaflets, 6 = severe disease and leaf defoliation, and 7= plant dead (Abbasi et al., 2002). Three plant replicates were used for each treatment. The experiment to track the in-planta population of Xp was repeated three times. In-planta population of Xa and Pc was enumerated in two of the three experimental sets. Plots of $\log_{10}$ (CFU/ cm^2) against time were generated to study the growth of each strain.

PAMP Triggered Immunity (PTI) Assay

The PTI cell death assay was performed as described in (Chakravarthy et al., 2009). Five to six weeks old FL 47R tomato plants were used for this study. They were placed in a growth chamber with ambient humidity, 12-hour light/dark cycle and temperature ranging from 28 to 25°C (day: night). *Xanthomonas perforans* AL65::Tn7-mre152 (Xp) bacterial strain was used as effector-

triggered susceptibility inducing "challenger" strain. *Xanthomonas arboricola* CFBP 6826::Tn7-mre160 (Xa) and *Pseudomonas* species 93B.260::Tn7-mre145 (Pc) were used as PTI inducers. Pc was cultured on King's B media. Xa was cultured on NYG media. All the strains were allowed to grow at 28°C for 48 hours. Next the cells from each strain were harvested separately in $MgSO_4$. The cell suspension was centrifuged to collect the cells and resuspended in $MgSO_4$. The centrifugation resuspension step was repeated twice. The strains were adjusted to a final O.D of 0.3 (CFU 1 x 10^8/ml) at 600 nm. The cell suspension was diluted 10 times using MgSO4 and the final concentration was 1 x 10^7 CFU /ml, which was used for infiltration for each strain. PTI inducer strains were infiltrated on leaves in a rectangular area with a 1 ml sterile syringe. Five plants were inoculated with Pc and five plants were inoculated with Xa. In each plant, 6 leaves were used for infiltration. 4 hours after the inducer infiltration (with PTI inducers), the leaves were infiltrated with Xp, in the same order of plants as the PTI induction was done. A point on the periphery of the first inoculation rectangle was be used as the center of the second inoculation rectangle. The plants were placed back inside the growth chamber and monitored for the appearance of cell death in the area which were challenged with Xp. Cell death inside the overlapping region of infiltration indicated a breakdown of PTI by Xp (Chakravarthy et al., 2009).

Fluorescence Microscopy

To observe the colonization pattern of Xp, Pc and Xa, 4-5 weeks old FL 47R tomato plants were co-inoculated with bacterial cell suspension containing ~1 X 10^6 CFU/ml of Xp + Xa + Pc mixed in 1: 1: 1 ratio and incubated under high humidity. Colonization of Xp, Pc and Xa was observed under fluorescent microscope on day 1, 3, 6, 9 post inoculation. Samples of leaf (~1cm^2) were mounted on glass slide and secured with mounting gel and cover slip. One field of leaf samples

were observed using 3 different filters of the fluorescent microscope. For Xa (with mTagBFP2), the chosen filter had excitation ability at 402 nm and detected the emission at 457 nm. For Pc (with mScarlet-1), the filter had excitation ability at 569 nm and detected the emission at 593 nm. Filter which could scan with excitation at 495 nm and detect emission at 512 nm was chosen for Xp (sGFP). Pictures of the same field, taken with 3 different filters, were combined with overlay tool associated to the microscope software. The samples were observed under 40X magnification, and 10 random fields were viewed per leaf sample. Both leaf surface and leaf apoplast was observed under the microscope.

Data Analysis

Data analysis and visualization was done using mixed linear model in R. Analysis of variance (ANOVA) were performed to evaluate significant difference among the growth pattern of various treatments. Treatments were used as fixed variable and experiments were used as random variable. All data used for ANOVA were normally distributed. Growth rate, carrying capacity and doubling time of each bacterial strain was calculated using ‘growthcurver’ (Sprouffske & Wagner, 2016) package of R.

Results

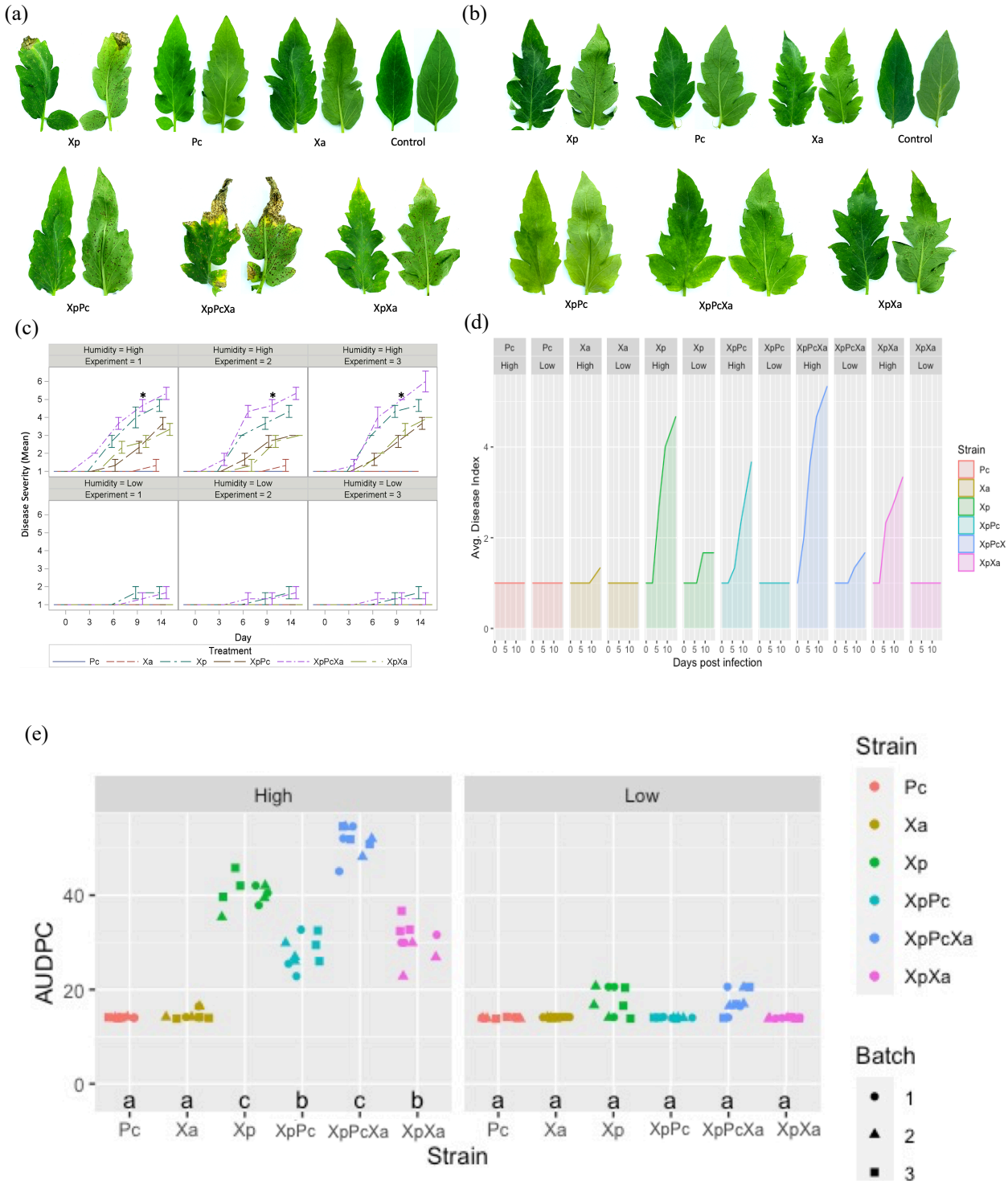


Figure 2. 1: Disease Severity under low and high humidity in individual and mixed inoculated plants. Four to five weeks old tomato (cv. FL47) plants were inoculated with ~1x

10^6 cfu/ml of Xp, Pc, Xa, Xp + Pc, Xp + Pc + Xa, Xp + Xa. (a) Disease severity on day 14 under high humidity (b) Disease severity on day 14 under low humidity. (c) Disease scale in individual inoculation. Vertical lines represent the 95% confidence limit. ANOVA repeated measurements (GLIMMIX model) was applied for the statistical analysis of the disease severity values. Treatment XpPcXa with * mark on day 9 is significantly higher disease severity compared to Pc, Xa, Xp + Pc, Xp + Pc + Xa, Xp + Xa according to Tukey's test of least significant difference ($P<0.05$) (d) Area under disease progress curve (AUDPC) for tomato plants co-inoculated with the different treatments (e) AUDPC raw values and mean with grouping letters (a, b, c) according to significant (P value<0.05) difference from linear mixed model with 95% confidence interval per treatments plotted from three different experiments.

Highest disease severity and highest area under disease progress curve is resulted by mixed infection caused by Xp, Pc and Xa together, compared to them alone

The disease severity of plants inoculated with Xp alone and in co-inoculation with Pc, Xa, or Pc + Xa was significantly higher ($p<0.05$) under high humidity compared to low humidity, with symptoms appearing as early as day 4 post-inoculation under high humidity versus 6 days post-inoculation under low humidity (Figure 2.1c). Plants inoculated with Xa, or Pc alone did not show any significant differences in disease severity between high and low humidity. Pc did not develop any visible symptoms under either high or low humidity condition throughout the 14 days of experiment (Figure 2.1a & b). Plants inoculated with Xa developed some symptoms only under high humidity after 9 days post inoculation. On day 9, under high humidity, plants co-inoculated with the three bacterial species Xp, Pc, Xa showed significantly higher mean bacterial leaf spot (BLS) disease severity ($p<0.05$) ranging from 5 to 6 on a disease scale, compared to Xp + Pc and

Xp + Xa, Pc and Xa showing disease severity between 1 to 2.5 to on the disease scale (Figure 2.1c). Under low humidity, there were no significant differences in disease severity among any of the treatments over the 14-day period.

In figure 2.1d, the disease progress curve of plants treated with Pc under high and low humidity did not increase, because no disease symptom was observed (in disease scale 1 = symptomless). For Xa, initially the disease progress curve did not increase, but under high humidity there was a slight increase at the end of the curve. Under high humidity, the symptoms caused by Xa were very less and took longer time to develop. Also, the lesions did not enlarge or spread. The disease progress curve of Xp under high humidity increased rapidly and the peak reached the 4.5 in the disease scale. The total area of disease increased rapidly. The disease severity curve of Xp under low humidity, had a delayed increase compared to high humidity and the curve increased slowly and remained static. It also covered less area compared to Xp under high humidity. The disease progress curve of XpPc under high humidity, increased gradually, followed by a rapid increase and it covered greater area compared to XpPc low humidity, which did not have any peak in the curve. The disease progress curve of XpPcXa under high humidity, increased sharply and had the highest peak and covered the highest area compared to XpPcXa low humidity curve and disease progress curve of all other treatments.

The visual representation of AUDPC (area under disease progress curve) values in Figure 2.1e, also indicated highest disease severity is developed under high humidity by mixed infection of Xp, Pc and Xa. Under low humidity there were no significant differences among the different treatments. Under high humidity, AUDPC values of Xp infected plants & XpPcXa infected plants were significantly higher than rest of the treatments. Even though XpPcXa infected plants had higher AUDPC than Xp infected plants, the difference was not significant. AUDPC values of

plants mix-inoculated with Xp + Pc and Xp + Xa, had no significant difference between them but both were significantly higher compared to plants treated with pure cultures of Pc and Xa.

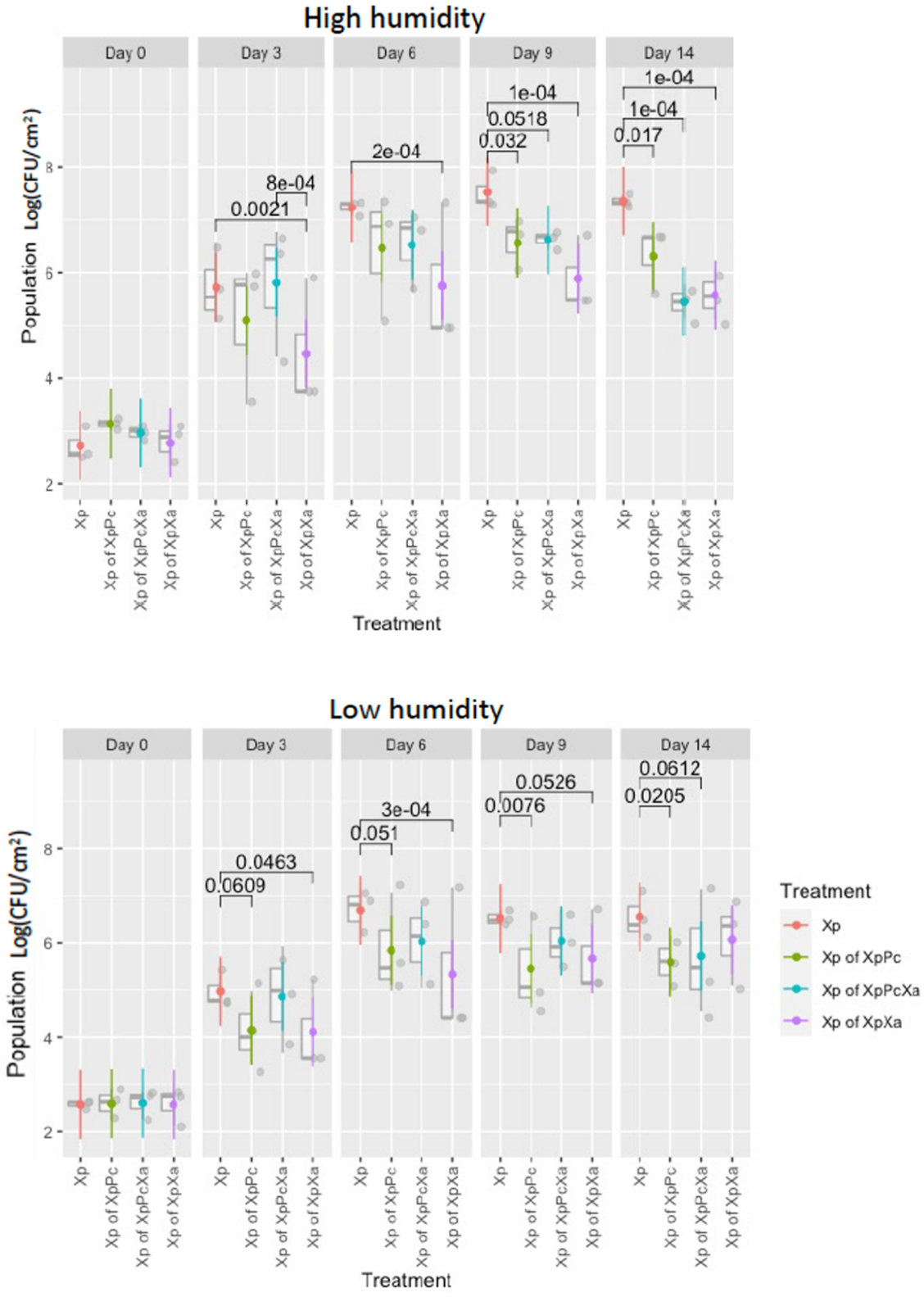


Figure 2. 2: Effect of Xa and Pc on Xp in-planta population. Four to five weeks old tomato (cv. FL47) plants were inoculated with ~1x 10^6 cfu/ml of Xp, Xp + Pc, Xp + Xa, Xp + Xa + Pc.

Growth of Xp population was evaluated from plants inoculated with the different treatments on day 0, day 3, day 6, day 9 and day 14 post inoculation on selective media. ANOVA (mixed linear model) was applied for the statistical analysis of the $\log_{10}$ cfu/cm^2 of Xp values. Significant differences ($P<0.05$) among the treatments, according to Tukey's test of least significant difference are depicted in the graph

Xp population is predominantly reduced by Xa as well as Pc under high humidity

Population of Xp individually and in different treatments (XpPc, XpPcXa and XpXa) was less than one log (log of cfu/cm^2) higher under high humidity compared to low humidity (Figure 2.2). Significant difference was observed in Xp population density between high and low humidity treatments on 9 dpi when Xp was inoculated alone and in presence of Pc. From 3 dpi (days post inoculation) till 14 dpi, presence of Xa significantly ($P<0.05$) lowered the population of Xp more than one log compared to Xp alone. On day 9 and day 14 post inoculation, combined presence of both Xa & Pc, also reduced the population of Xp compared to when Xp was alone. Results show that Pc was able to reduce Xp population significantly starting from only 9 dpi, whereas Xa could reduce Xp population from as early as 3 dpi. It indicates that, under high humidity, Xa had more influence on overall *in planta* growth of Xp from the beginning of the infection, while Pc influences Xp population growth later during the disease progression.

Under low humidity Pc lowers Xp population

We observed that under low humidity, on day 3, 6, 9, 14 days post inoculation, Xp population was significantly ($P<0.05$) ~ 1 log lower in presence of Pc compared to Xp being present individually (Figure 2.2). Therefore, Xp population was reduced by Pc from as early as 3 dpi under low

humidity conditions. Such reductions in population size were observed under high humidity in the later stages of disease, i.e., day 9 post-inoculation, indicating that humidity plays an important role in influencing the outcomes of Xp-Pc interactions during early stages of disease development.

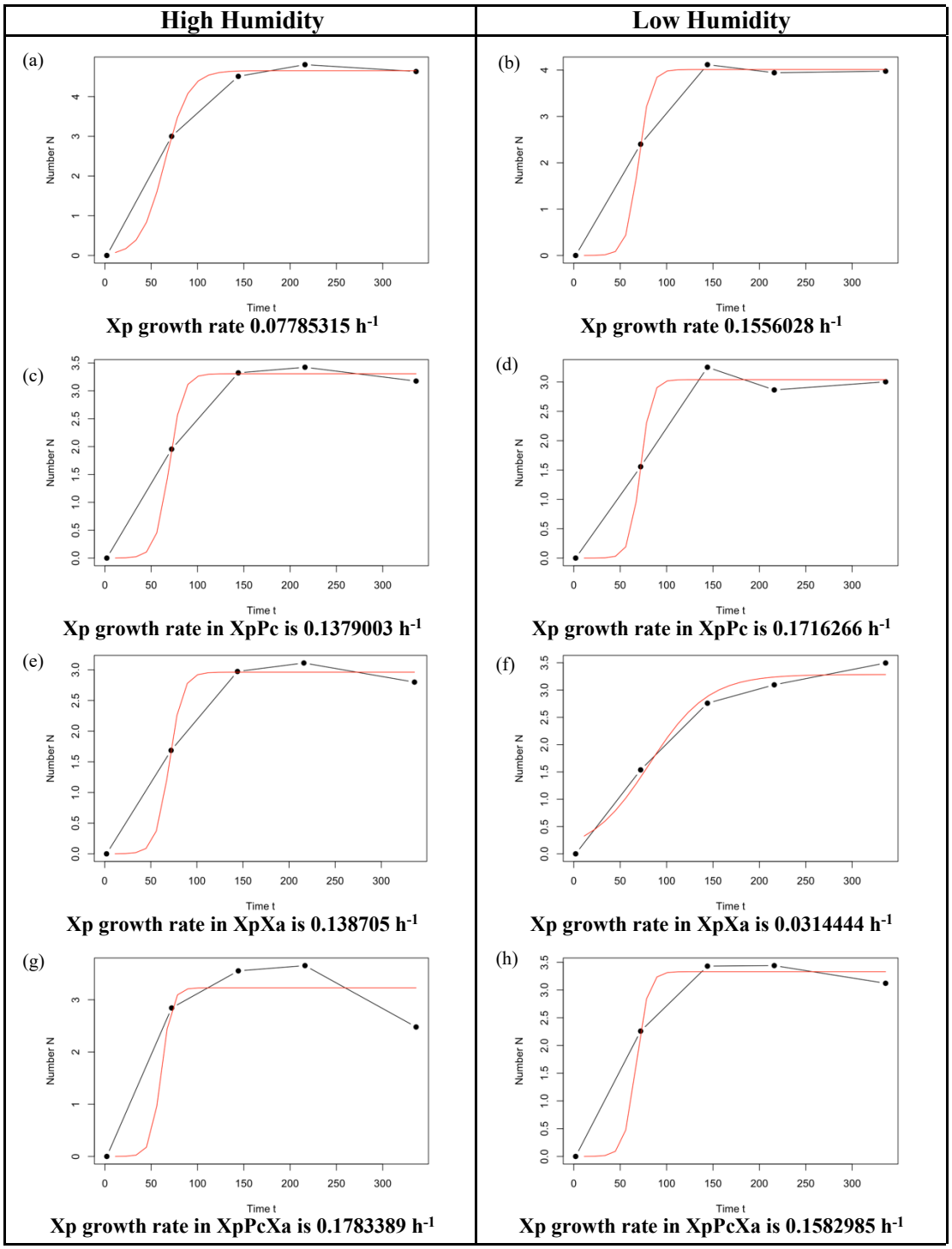


Figure 2. 3: In planta growth rates of Xp. Population size at each time point is represented by 'N' in Y axis and X axis is presenting time in terms of hours. XpPc – Xp + Xa, XpXa – Xp + Xa, XpPcXa – Xp + Pc + Xa

Table 2. 4: Growth rate, doubling time and host carrying capacity of in planta Xp population

Treatment	Growth rate	Doubling time	Carrying capacity
Xp High humidity	0.07785315 h^{-1}	8.9 hours	4.65 $\log_{10}$ cfu/cm^2
Xp Low humidity	0.1556028 h^{-1}	4.45 hours	4.01 $\log_{10}$ cfu/cm^2
Xp of XpPc High humidity	0.1379003 h^{-1}	5.03 hours	3.31 $\log_{10}$ cfu/cm^2
Xp of XpPc Low humidity	0.1716266 h^{-1}	4.04 hours	3.04 $\log_{10}$ cfu/cm^2
Xp of XpXa High humidity	0.138705 h^{-1}	5 hours	2.96 $\log_{10}$ cfu/cm^2
Xp of XpXa Low humidity	0.0314444 h^{-1}	22 hours	3.29 $\log_{10}$ cfu/cm^2
Xp of XpPcXa High humidity	0.1783389 h^{-1}	3.89 hours	3.23 $\log_{10}$ cfu/cm^2
Xp of XpPcXa Low humidity	0.1582985 h^{-1}	4.38 hours	3.33 $\log_{10}$ cfu/cm^2

Under high humidity Xp population had lowest growth rate and highest carrying capacity in absence of Pc and Xa, but under low humidity Xp population had the lowest growth rate in presence of Xa and highest doubling time.

Growth rate, doubling time and the carrying capacity for Xp population was calculated for all treatments under low and high humidity conditions with Xp population in single and mixed infections (N or $\log_{10}$ cfu/cm^2) on the day of inoculation and (day 0) 3, 6, 9 and 14 dpi. Under high humidity, growth rate of Xp was lowest when plants were inoculated with pure inoculum of Xp, but it also had the highest carrying capacity (4.65 $\log_{10}$ cfu/cm^2), meaning the maximum number of bacteria the leaf surface could support was higher compared to all other treatments. Plants inoculated with pure culture of Xp also had the second longest doubling or generation time of 8.9 hours.

Presence of Pc and presence of Xa increased Xp growth rate from 0.07785315 h^{-1} to 0.1379003 h^{-1} and 0.138705 h^{-1} respectively (Figure 2.3a, 2.3c, 2.3e). Presence of Pc and Xa also decreased the carrying capacity and reduced the generation time to 5.03 hours & 5 hours respectively. The leaf surface had the lowest carrying capacity (2.96 $\log_{10}$ cfu/cm^2) for Xp under high humidity in presence of Xa. It indicates that presence of Xa under high humidity can be unfavorable for Xp population. We observed the highest growth rate of Xp under high humidity in presence of Xa and Pc combinedly, which was 0.1783389 h^{-1} (Figure 2.3g). Combined presence of Xa and Pc, also enabled Pc to achieve the shortest doubling time (3.89 hours). Under high humidity, Xp +Pc + Xa co-inoculated plants had reduced carrying capacity of Xp (3.23 $\log_{10}$ cfu/cm^2) compared to when plants were inoculated with only Xp. Presence of Pc and Xa increased the growth rate of Xp, but it also reduced the population of Xp (Figure 2.2) which corelates with the reduced carrying capacity for Xp.

Under low humidity growth rate of Xp was almost similar in presence of both Pc and Xa combinedly compared to Xp alone, but combined presence of Pc and Xa, lowered the carrying capacity and doubling time for Xp. Presence of Pc increased the growth rate of Xp slightly and reduced the doubling time. Presence of Pc also reduced the carrying capacity for Xp compared to presence of Xp alone and this observation corelated with the result that Xp population was reduced under low humidity by Pc from as early as day 3 post inoculation. Presence of Xa, reduced the growth rate of Xp drastically from 0.1556028 h^{-1} to 0.0314444 h^{-1} and the doubling time of Xp increased to 22 hours which was the highest doubling time required for Xp among all other treatments. Presence of Xa, also lowered the carrying capacity for Xp from 4.01 $\log_{10}$ cfu/cm^2 to 3.29 $\log_{10}$ cfu/cm^2. Lower Xp growth rate and the longest doubling time under low humidity in

presence of Xa can be considered as the cause of lower Xp population in plants inoculated with Xp + Xa (Figure 2.2).

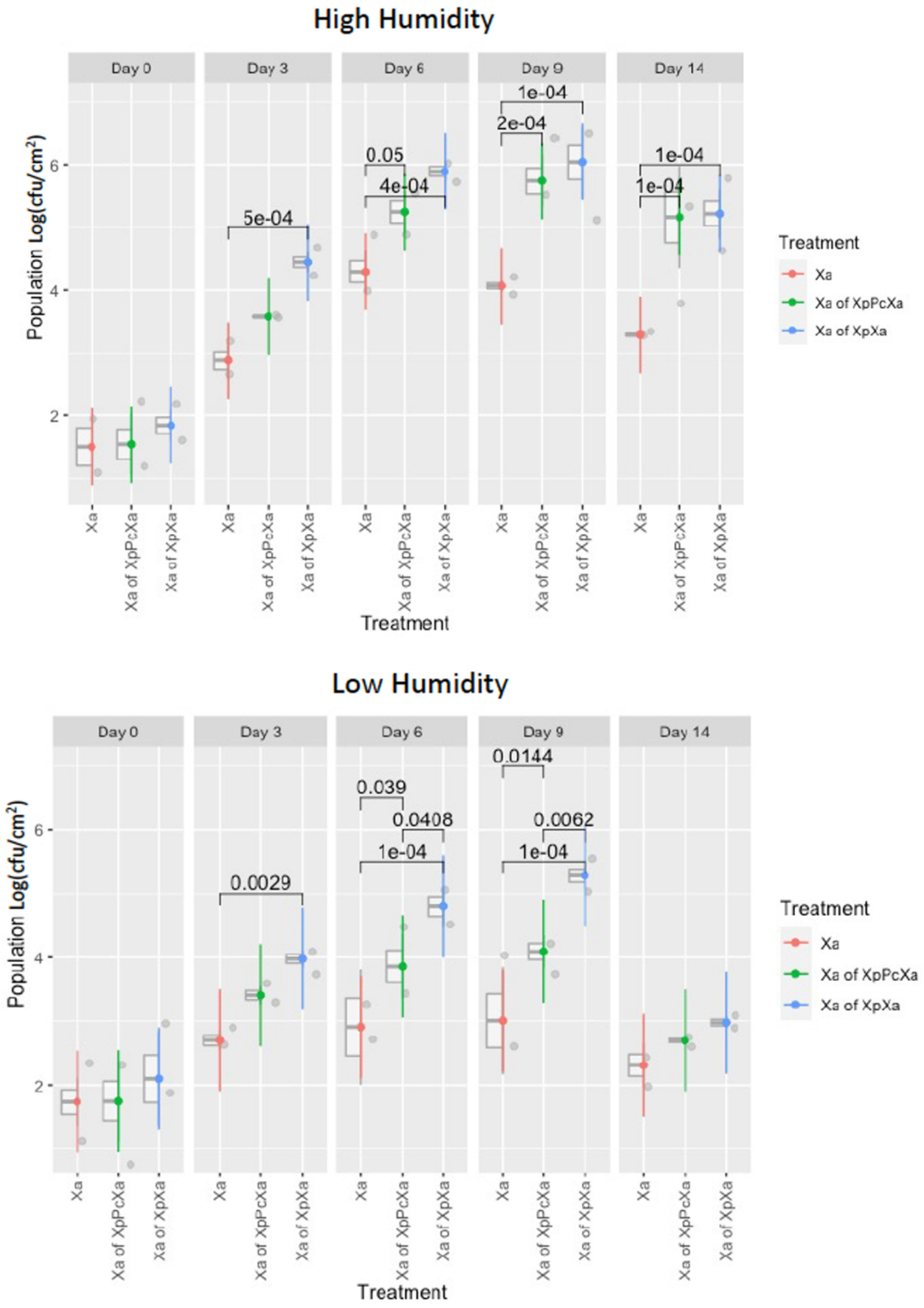


Figure 2. 4: Effect of Xp and Pc on Xa in-planta population. Four to five weeks old tomato (cv. FL47) plants were inoculated with ~1x 10^6 cfu/ml of Xa, Xp + Pc + Xa, Xp + Xa and incubated in growth chamber under high. Growth of Xa population was evaluated from plants

inoculated with the different treatments on day 0, day 3, day 6, day 9 and day 14 post inoculation on selective media. ANOVA (mixed linear model) was applied for the statistical analysis of the $\log_{10}$ cfu/cm^2 of Xp values. Significant differences ($P<0.05$) among the treatments, according to Tukey's test of least significant difference are depicted in the graph.

Xa population significantly increases in presence of Xp under high & low humidity conditions

Higher humidity did not significantly change Xa population compared to low humidity when Xa was alone. Xa population was significantly higher under high humidity compared to low humidity, in presence of Xp or combined presence of Xp and Pc on 14 dpi (Figure 2.4). Combined presence of Xp and Pc under high humidity, also significantly promoted the growth of Xa compared to low humidity on 9 and 14 dpi. Under high humidity, Xp significantly ($P<0.05$) increased the population of Xa compared to when Xa was alone, from 3 days post inoculation till 14 days post inoculation. Population of Xa was significantly ($P<0.05$) increased in the presence of Pc and Xp combinedly, under high humidity, from 6 days post inoculation to 14 days post inoculation. No significant difference was observed in Xa population between the treatments Xp + Xa and Xp + Pc + Xa. It indicated that presence of Pc did not influence population of Xa under high humidity. Population of Xa was ~1 log higher under high humidity compared to low humidity when plants were inoculated with Xa alone.

Similar to high humidity conditions, under low humidity, Xp significantly ($P<0.05$) promoted the growth of Xa compared to when Xa was alone, on 3, 6, 9 days post inoculation. We also observed that on day 6 and day 9 post inoculation, population of Xa increased significantly ($P<0.05$) in presence of Pc and Xa together, compared to Xa alone. Under low humidity on 6 and 9 dpi, Xa

population was significantly higher in presence of Xp, compared to Xa population in presence of both Xp & Pc combined. Therefore, Xa colonizes better with help of Xp.

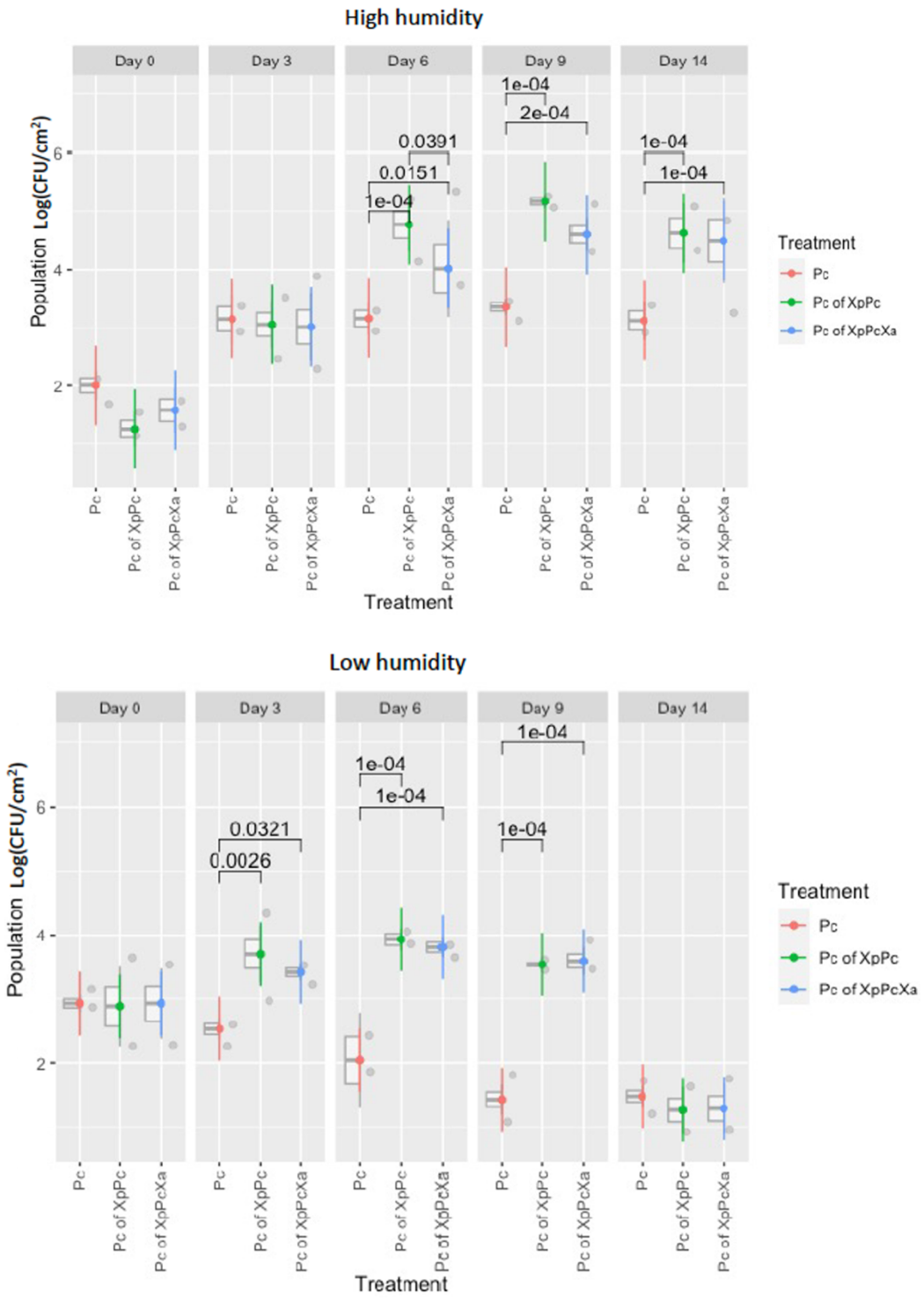


Figure 2. 5: Influence of Xp and Xa on Pc in-planta population. Four to five weeks old tomato (cv. FL47) plants were inoculated with ~1x 10^6 cfu/ml of Pc, Xp + Pc, Xp + Pc + Xa. Growth of Pc population was evaluated from plants inoculated with the different treatments on day 0, day 3, day 6, day 9 and day 14 post inoculation on selective media. ANOVA (mixed linear

model) was applied for the statistical analysis of the $\log_{10}$ cfu/cm^2 of Xp values. Significant differences ($P<0.05$) among the treatments, according to Tukey's test of least significant difference are depicted in the graph.

Population of Pc increases in the presence of Xp and Xa under high humidity

Population of Pc was tracked from plants co-inoculated with Pc, Xp + Pc, and Xp + Pc + Xa (Figure 2.5). Higher humidity significantly increased the Pc population compared to low humidity, at the later stage of disease progression 9 and 14 dpi, when Pc was inoculated by itself. High humidity also increased Pc population compared to low humidity in presence of Xp on 9 dpi. Under high humidity, from 6 dpi till 14 dpi, combined presence of Xp & Xa, significantly ($P<0.05$) increased the population of Pc, compared to when Pc was alone. A significant increase ($P<0.05$) in Pc population was also observed under high humidity in presence of Xp on day 6, day 9 and day 14 post inoculation. A significant difference in Pc population was observed when compared in presence of Xp + Pc and Xp + Pc + Xa. It indicates that both Xa and Xp plays a role in increasing the Pc population under high humidity. Under high humidity, Pc population did not reach its death or decline phase as quickly as under low humidity.

Under low humidity, significant increase ($P<0.05$) in Pc population was also observed in presence of Xp and in combined presence of Xp + Xa. Absence of Xa did not cause any significant difference within the Pc population. Under low humidity, on day 14 post inoculation, Pc population was under 2 $\log_{10}$ cfu/cm^2 for all treatments, which was not observed under high humidity. Thus, Pc can possibly survive better under higher humidity and in presence of Xp.

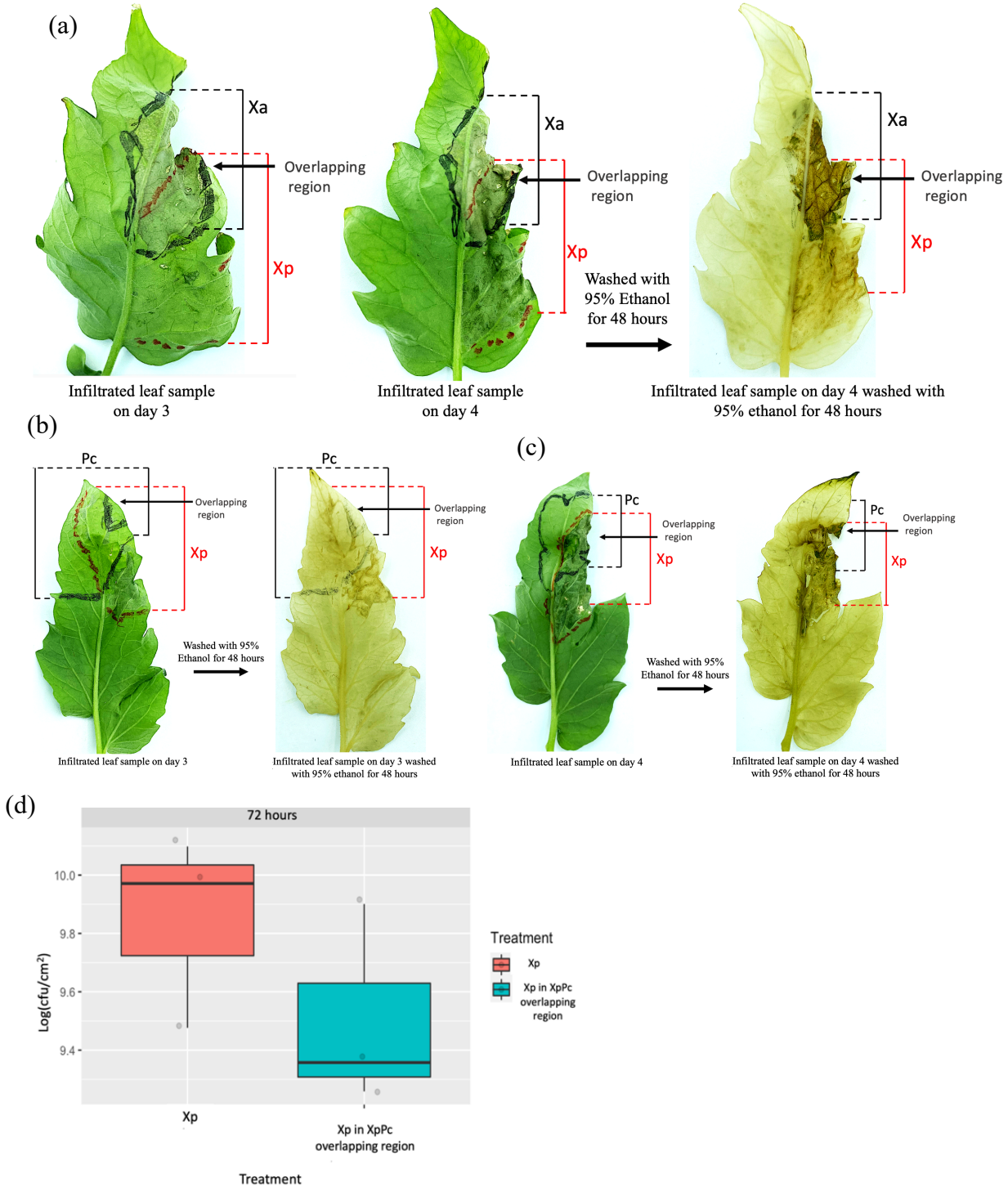


Figure 2. 6: PTI assay. Tomato leaves were infiltrated with Xa or Pc to induce PAMP triggered immunity (area between the two black lines), and 4 hours later, the leaves were challenged with Xp (area between the two red lines). (a) On day 3, water soaking or cell death symptom by Xa

was observed in leaf area infiltrated with Xa and the overlapping region infiltrated with both Xa and Xp. On day 4, cell death was also observed in the area infiltrated with only Xp. Leaf with symptom from day 4 was ethanol washed to visualize the cell death better. (b) On day 3, water soaking or cell death symptoms by Xp were observed in leaves infiltrated with Pc and Xp. No cell death was observed in the overlapping regions. Cell death was clearly visible after ethanol wash (c) On day 4 cell death was observed in the overlapping area which indicated a breakdown of PTI in the leaves infiltrated with Pc and Xp. (d) Day 3 population of Xp from the overlapping region infiltrated with Xp + Pc, and population of Xp from the area infiltrated with Xp only. Vertical lines represent standard error bars. T-test was done on log10 cfu/cm2 values and difference between two treatments were not significant ($P<0.05$). Concentration of each inoculum was 1 x 107 CFU /ml

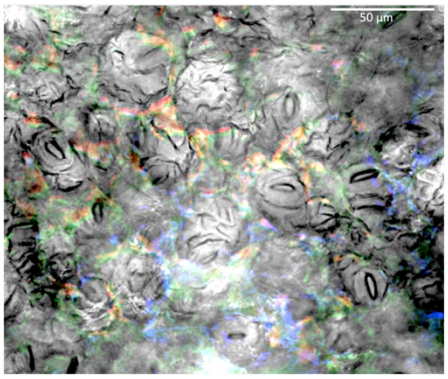
Xa induces cell death response upon infiltration into the apoplast

Leaves infiltrated with Xa and Xp were monitored for the development of disease symptom or cell death. On day 3, the area of the leaf infiltrated with Xa, and the overlapping region where both Xp and Xa were infiltrated, both developed symptoms of cell death (Figure 2.6a). No symptoms were observed in the area which was infiltrated with only Xp. After 24 hours, symptom of cell death was also observed in the Xp infiltrated region on day 4. Next the leaf was washed with 95% ethanol for 48 hours to clearly visualize the cell death symptoms. Since this cell death response was observed after 3 days post-inoculation, it may be a susceptibility related cell death, as opposed to cell death resulting from PAMP or DAMP (Damage-Associated Molecular Patterns) triggered immunity.

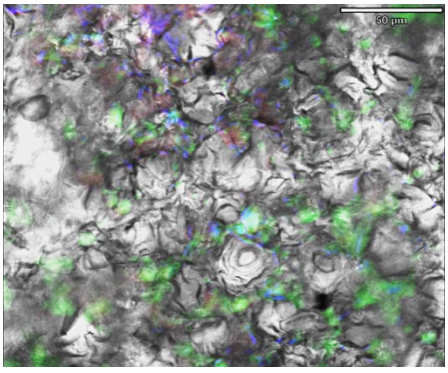
Pre-inoculation of tomato leaves with Pc induces PAMP triggered immunity in the host and delays cell death caused by Xp

Pc and Xp infiltrated leaves were monitored every day for symptom development. Pc was infiltrated in the area between two black lines. Xp was infiltrated in the area between two red lines. On day 3, cell death was observed in the area infiltrated with only Xp, (Figure 2.6b). There were no symptoms in the overlapping region of the leaf infiltrated with both Pc and Xp or Pc alone. After the next 24 hours, cell death symptoms developed in the overlapping region. Thus, a delay in cell death symptoms by Xp was observed in the overlapping area where prior Pc infiltration was carried out before challenge inoculation with Xp, indicating delayed susceptibility response in presence of Pc. Pc was able to induce PAMP-triggered immunity within 4 hours and caused a delay in the effector triggered susceptibility by Xp, After 24 hours, Xp was able to overcome or suppress the PTI cell death response was observed in the overlapping area (Figure 2.6c). The lack of cell-death incited in presence of Pc alone indicates that Pc lacks effector or virulent proteins which could have suppressed the PTI of the host. PTI is induced earlier than ETI (effector triggered immunity). To investigate if the population of Xp was lower by PTI in the overlapping region infiltrated with both Pc and Xa, we compared the Xp population of from overlapping region with the region infiltrated with only Xp (Figure 2.6d). Population of Xp from the overlapping region was half a log lower than Xp population from the region infiltrated with only Xp. However, the difference in the population was not significant. Even though the decrease in the Xp population by PTI was not significant, PTI raised by Pc did delay Xp-induced cell death susceptibility response by 24 hours.

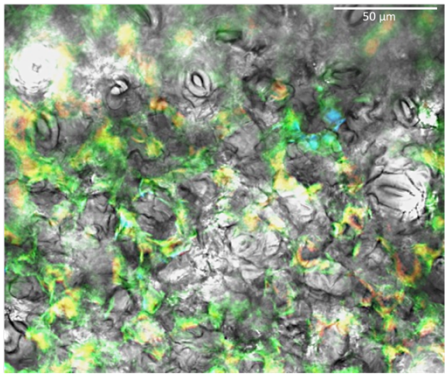
Leaf surface



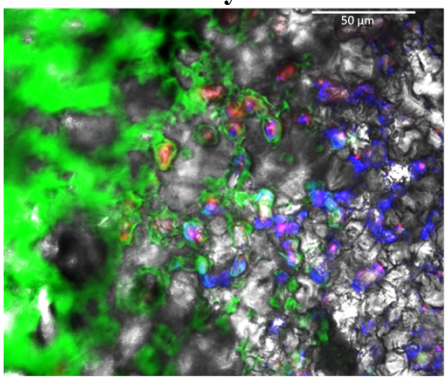
Day 1



Day 3

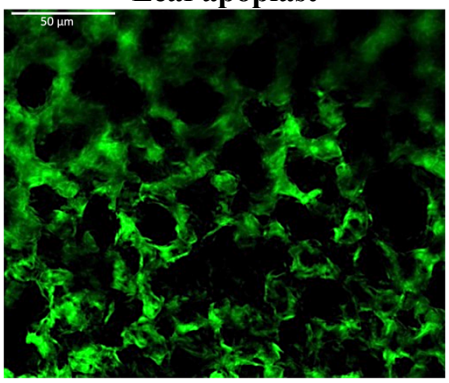


Day 6

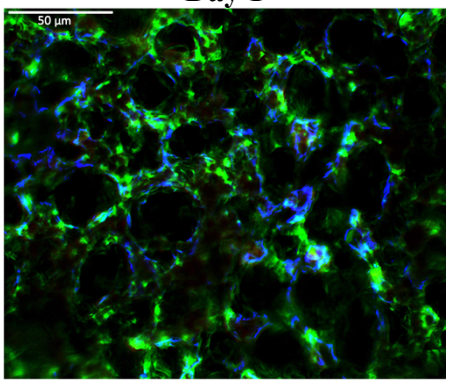


Day 9

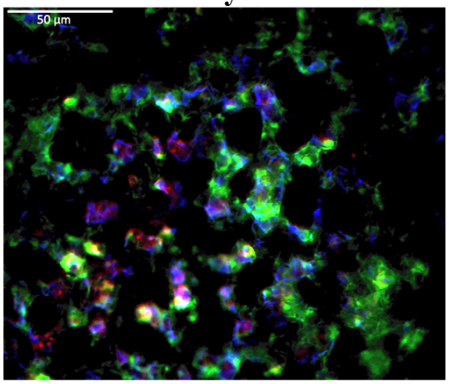
Leaf apoplast



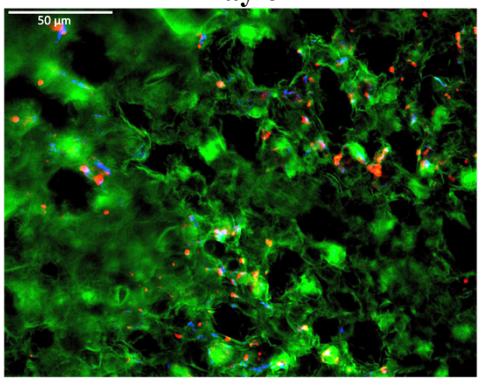
Day 1



Day 3



Day 6



Day 9

Figure 2. 7: Colonization of Xp, Pc and Xa on leaves of tomato plants inoculated with the three bacterial species on 1, 3, 6, 9 days post inoculation. Four to five weeks old tomato (cv. FL47) plants were co-inoculated with ~1x 10^6 cfu/ml of Xp, Pc and Xa. Xp expressed sGFP2 fluorescent protein and appeared green under the microscope. Xa expressed mTagBFP2 fluorescent protein and appeared blue, Pc expressed mScarlet-I fluorescent protein and appeared red under the microscope. Leaf samples were observed under 40X magnification. Pictures were taken along the Z stack. Leaf surface or top 20,165 μm, bottom 20,174.4 μm, scale bar = 50μm

Xp dominates the leaf apoplast in plants co-inoculated with Xp, Pc, and Xa, whereas Pc and Xa are epiphytes that survive better in presence of Xp

To investigate the colonization pattern of Xp, Pc and Xa, 4 to 5 weeks old tomato plants were co-inoculated with bacterial cell suspension containing ~1 X 10^6 CFU/ml of Xp + Xa + Pc mixed in 1: 1: 1 ratio and incubated under high humidity. The leaf samples from the co-inoculated plants were examined under the fluorescent microscope on 1-, 3-, 6- and 9-days post inoculation (Figure 2.7). On day 1 we observed that the leaf apoplast was predominantly colonized by Xp. Xp colonized in grooves or borders around the cells although there were no disease symptoms developed in the leaves by 1 dpi. Even though we did not detect Pc and Xa in the apoplast, signals of Pc and Xa were detected in the leaf surface, and throughout the leaf surface presence of Xa or Pc were visibly less compared to Xp during early colonization. On day 3 post inoculation, along with Xp, Xa was also observed to colonize in the cell grooves of leaf apoplast as well as leaf surface. However, presence of Pc was observed to be comparatively lower than Xa and Xp in the apoplast. Disease symptoms developed on the co-inoculated leaves by day 4 post inoculation. On 6-day post inoculation, in both leaf surface and apoplast, association of Pc and Xa with Xp was

visibly higher compared to day 3 leaf samples. Aggregates of Xa, Pc and Xp were observed around cell grooves. By day 9, the leaves of the co-inoculated plants had high disease severity with many spots with coalescence on many leaflets. Distribution of Xp was high and aggregates of Xp was observed throughout the sample. Xp or Pc or Xa were not observed in the areas of the leaf with disease symptoms and cell death or dead leaf tissue. Presence of Xa and Pc were also observed around the grooves of the cells on 9 dpi.

Discussion

In this study, we observed that mixed infection of Xp and two other weak or opportunistic pathogens, Xa and Pc, leads to highest disease severity under high humidity in tomato plants when compared to single infections, yet *Xanthomonas perforans* population was significantly lower in the mixed infection compared to single infection. We assessed influence of humidity on in planta disease dynamics and population dynamics in mixed infection and infection by individual strains. To understand the contribution of each member, we enumerated population of individual strain using selectable antibiotic marker. Interactions among each member in the mixed infection and their influence on colonization patterns upon dip-inoculation of plants were studied using epifluorescence microscopy and finally, involvement of host defense response in influencing mixed infection outcome was investigated.

It has been observed that *Xanthomonas perforans* requires high humidity for enhanced disease development and tropical or subtropical regions with high humidity has been previously identified to have the ideal condition for them (Abrahamian et al., 2021; Carvalho et al., 2019; Obradovic et al., 2008) and results of our study substantiate this observation but higher humidity could not significantly induce disease severity in plants inoculated with either only Pc or only Xa, although

it did increase the disease severity of Xa inoculated plants at the late stages of disease progression. Different pathovars of *Xanthomonas arboricola* bacterial strain has been observed to cause disease in humid and warm condition (Kałużna et al., 2021; Lamichhane, 2014; Lamichhane & Varvaro, 2014). This observation resembles the opportunistic pathogenic nature of Xa during its interaction with tomato plants. Comparable AUDPC values between single Xp infection and mixed infection at high humidity observed in our study indicates that presence of other opportunistic pathogens does not result in change in overall disease severity. We also observed that high humidity increased Pc population significantly at the last stage of the disease which proved that high humidity is beneficial for Pc. It has also been previously documented that humidity helps *Pseudomonas cichorii* survive better and disease development (Cambra et al., 2004; Janse, 1987). *Pseudomonas cichorii* has been recorded to cause pith necrosis in tomato plants (Trantas et al., 2013). Several commensal *Pseudomonas* species like *Pseudomonas marginalis*, *Pseudomonas putida*, *Pseudomonas protegens*, *Pseudomonas citronellolis* have been observed to cause increased disease severity of pith necrosis in tomato when coinfected with *Xanthomonas perforans.* However, in our study when we examined for pith necrosis symptoms, we did not observe any vascular browning in the plants that were inoculated with either Pc or both Xp & Pc (Aiello et al., 2017). In tomato fields of southeastern United States, bacterial leaf spot disease outbreak is common due to the subtropical climate and frequent rainfall which creates a highly humid environment (Abrahamian et al., 2019). From results of our study, we observed that presence of Xp, increased the Pc and Xa population. Therefore, Pc and Xa can be considered as poor colonizers because Pc cannot produce the principal cell wall degrading enzymes like cellulase, pectate lyase and protease and both Pc and Xa do not have the complete T6SS (Bernal et al., 2018) and enough Type 3 virulence effectors. In a study done by Nix et al., similar results were observed when

responses of 2 epiphytic microbial species (*Rhodotorula glutinis* & *Cryptococcus laurentii*) were investigated upon foliar infection by the pathogen *Rhizoctonia solani*. The abundance of the two epiphytic yeast species in *Rhizoctonia solani* infected plants, were always greater than healthy plants (Nix et al., 2009).

So, we can speculate that in fields higher humidity might be more advantageous for survival or host colonization by opportunistic species like *P. cichorii* and *X. arboricola*, when another dominant pathogen like *Xanthomonas perforans* is present because Xp and can cross the host barrier and makes it less difficult for the weak pathogens to enter the host. It has been documented that ability of a pathogen to persist and cause disease in a host plant is usually the exception, not the rule (Staskawicz, 2001). We can also predict that mixed infection by the bacterial leaf spot pathogen and its co-occurring bacterial species might not significantly increase the disease severity or the crop loss. In mixed infection, susceptibility response could be a result of Xp-induced cell death and opportunistic infection by Xa, Pc and thus cell death.

Although we observed higher disease severity in presence of mixed infection, overall population of Xp was reduced by the presence of Pc or Xa under high humidity conditions. Similarly, a study on the bacterial leaf streak pathogen of wheat *Xanthomonas translucens pv. translucens*, revealed that bacterial epiphytes of wheat phyllosphere could reduce both disease severity and the population of *Xanthomonas translucens pv. translucens,* when the epiphytes were inoculated 48 hours prior to the pathogen strain *X. translucens pv. translucens.* However, they observed that nutrient overlap or antibiosis was not the reason behind decreased pathogen population (Stromberg et al., 2000). In our study we observed that in Xp + Pc or Xp + Pc + Xa co-inoculated plants, Pc significantly reduced Xp population under high humidity at the later stages of infection, but Pc

could do the same under low humidity from the early stages of the infection. It indicates that during initial stage of disease development high humidity plays a key role in regulating the interaction between Xp and Pc. Also, under high humid condition Pc can only influence Xp population later during the disease progression. However, the growth rate of Xp increased and Xp doubling time reduced, in presence of Pc, under both low and high humidity. The increased growth rate was possibly a response of Xp to the competition experienced due to presence of other species. Bacterial growth has been observed to be related to its survival, when an induvial bacterial species attempts to withstand predation by protozoa, in an environment with mixture of other bacterial species (Gurijala & Alexander, 1990). In a study by Mallory et al., it was noticed that microbial predators can also eliminate non-growing bacterial species, in presence other bacterial species with higher density (Mallory et al., 1983). The carrying capacity for Xp also reduced in presence of Pc under both low and high humid condition. 'Carrying capacity' of an environment is defined by the maximum number of individuals that can be supported by the environment (McArthur, 2006). Typically the known key factor that determine foliar carrying capacity is the availability of nutrients (Remus-Emsermann et al., 2012). It is possible that presence of Pc reduced the available nutrition utilized by Xp and resulted in lower Xp population and carrying capacity. This observation corelates with the nutritional profile similarity studied in chapter 3, where we observed high nutritional similarity among the three bacterial species, which indicates a nutritional overlap. Using microscopy, we also observed that during the early days of colonization, Pc was detected on the leaf surface but not in the apoplast and Xp was prevalent in the apoplast during this period. This observation corelated with our population data of early infection days (3dpi) where Xp population was high (~ 6 $\log_{10}$ cfu/cm^2) when Xp was co-inoculated under high humidity with both Pc and Xa and population of Pc was ~ 3 $\log_{10}$ cfu/cm^2. After symptoms started developing in the

leaves, we started detecting the presence of Pc in the apoplast as well as leaf surface. Pc colonized the cellular grooves of apoplast along with Xp. This resembled the Pc population data where Pc population was higher (~ 4.5 $\log_{10}$ cfu/cm^2) in presence of Xp during the later stages of infection. Therefore, Pc was able to colonize the apoplast better in presence of Xp. Pc is a poor colonizer but when in presence of Xp, Pc can colonize the host plant better. Observation of Pc colonizing the apoplast and leaf surface along with Xp also suggests niche overlap which was also indicated from the nutritional similarity evaluated from our in-vitro studies. To further understand the reason behind Xp population reduction by Pc, we conducted PAMP triggered immunity assay and investigated the role of plant's host immune response in this interaction. Plants have two layers of defense against pathogens. First one is Pathogen-Associated Molecular Pattern (PAMP)-Triggered Immunity (PTI) and the second one is Effector-triggered immunity (ETI) (Andolfo & Ercolano, 2015). Pathogens that possess virulent factors or effectors, are capable of suppressing PTI, and these pathogens can cause disease. Successful bacterial plant pathogens can inject virulence or effector proteins into the cytoplasm by type III secretion system. These effector proteins of the pathogen enter the host plant cell and are able to modulate host vesicle trafficking, which creates an environment in the host suitable for the survival of the pathogenic bacteria (Newman et al., 2013; Nomura et al., 2006). Plants that possess resistance genes against these effectors, can induce Effector-triggered immunity (ETI). Dominant bacterial leaf spot pathogen *Xanthomonas perforans*, suppresses PAMP- triggered immunity (PTI) and colonizes the plant host by inducing effector-triggered susceptibility (Potnis et al., 2014). We observed that in presence of Pc, effector triggered susceptibility by Xp is delayed for 24 hours. It indicated Pc lacks of effectors to suppress PAMP triggered immunity. The PTI induced by Pc also caused a slight (half a log) decrease in Xp population, where both Xp and Pc were infiltrated. The PTI induced in host by Pc is not meant to

stay restricted but also affect the neighboring host cells which is possibly restricting the Xp colonization and delaying susceptible response by Xp, but Xp could overcome the PTI even though it was delayed. Pc more likely lacks ability to suppress PTI as indicated by genome analysis data (Chapter 3) where we observed that Pc can produce only one type III effector. Previous reports show that *Pseudomonas syringae* pv. tomato produces a protease (AprA) which suppresses recognition of a PAMP (flagellin) by the host plant by cleaving it. Therefore, proteases produced by pathogens protect them from plant's primary defense mechanism like PTI (Figaj et al., 2019). In our study we observed that Pc lacks extracellular protease activity (chapter 3). Absence of protease enzyme in Pc corelates with induction of PTI in the host plant. We can conclude that several factors like host immunity, nutritional similarity, spatial competition, and high humidity contributed Pc to be able to reduce population of Xp.

Our study also exhibited that in plants co-inoculated with Xp + Xa or Xp + Pc + Xa, presence of Xa can also reduce population of the dominant pathogen Xp. Presence of Xa could significantly reduce Xp population starting from the early stages of infection till end, under both high and low humid condition. In contrast to this, presence of Pc could only significantly reduce Xp population throughout the disease progression under low humidity only. Therefore, it is possible that, compared to Pc, presence of Xa has more influence on overall in planta growth of Xp population under high humidity, from the beginning of the infection. Under high humidity, presence of Xa had increased growth rate and reduced doubling time. The higher growth rate and reduced doubling time can be an outcome of the effort to survive and maintain its population density (Gurijala & Alexander, 1990; Mallory et al., 1983), similar to what we observed in Xp population in presence of Pc. During the entire disease progress, we observed lowest population of Xp under both low

and high humidity in presence of Xa, which correlates with the carrying capacity for Xp in presence of Xa, which was lower than Xp alone under low humidity, and lowest among all other treatments under high humidity. In chapter 3, studying the nutritional profile of Xp and Xa revealed that nutritional similarity (NOIc) between Xp and Xa is relatively higher than others, which is 0.96. The highest nutritional similarity between Xp and Xa is possibly the reason for the lowest carrying capacity of Xp, in presence of Xa (Mercier & Lindow, 2000). In contrast to high humidity, presence of Xa did not increase the growth rate of Xp under low humidity and surprisingly, Xp had the lowest growth rate and highest doubling time. A potential reason can be assumed that under high humidity bigger influence of Xa, from the beginning of the infection, on the overall in planta growth of Xp population. While observing the co-inoculated plants to understand colonization pattern of the three bacterial species, during the early stage of infection (24 hours post inoculation), Xa was detected only in the leaf but not in the apoplast. After the initial stage (72 hours later), along with Xp, presence of Xa was detected both in the cellular grooves of the apoplast and leaf surface. In this period Xp was most prevalent in apoplast with highest spatial distribution and Xa was the 2nd most prevalent. This observation correlated with our *in planta* population assay results, where an increase in the Xa population was observed after 72 hours in presence of Xp. Day 9 post inoculation, as disease progressed more, and bacterial spots became bigger in the co-inoculated leaves we observed that leaf tissue under the microscope was dominated by Xp in both apoplast and leaf surface area.

The visual confirmation of co-colonization of Xp and Xa correlated with our suspected nutritional similarity or niche overlap between Xp and Xa. Xa can colonize the host plant better in presence of Xp, compared to Xa alone. The microscopy observation also indicated a possibility that Xa, Xp and Pc might also form mixed species biofilms, which help bacteria to survive better and withstand

external adversities (Rickard et al., 2003; Yang et al., 2011). However, more studies need to be done to observe the biofilm formation capability of these bacterial strains. In PAMP triggered immunity assay we observed cell death caused by Xa, and Xa did not delay cell death development by Xp. The cell death caused by Xa might have been caused by effector triggered susceptibility related cell death rather than PAMP (Pathogen-associated Molecular patterns) or DAMP-(Damage-Associated Molecular Patterns) triggered immunity, because cell death was observed after 72 hours. It is also possible that the cell death caused by Xa might have caused by some unknown mechanism. Another possibility is Xa managed to suppress PTI response by delivering effector proteins inside the host plant cell and created an environment for itself to survive inside the host. AvrBs2, XopZ, XopAL1, XopAW, HrpW, AvrA1are the type 3 effectors detected in Xa. However, we do not know the function of the effectors or to what extent they might have contributed. From these results, we can conclude that higher humidity is beneficial for Xa to reduce the Xp population and high similarity in the nutritional profile of Xp and Xa is one of the reasons that reduces the carrying capacity for Xp and reduces Xp population. Xp had a high growth rate in presence of Xa under high humidity probably as a survival response or as an effort to maintain its population density.

Overall, we can conclude that fitness of the dominant bacterial leaf spot pathogen *Xanthomonas perforans* population depends on the presence of its co-occurring bacterial species *Pseudomonas cichorii* and *Xanthomonas arboricola*, and high humidity. Humidity gives advantage to these two weak and opportunistic bacterial species to colonize better and reduce population of Xp. These three bacterial species probably compete for resources and spatial distribution in the leaf phyllosphere which in turn results in lower carrying capacity and lower population of Xp. The

dominant pathogen Xp, colonizes the leaf apoplast better. Xa and Pc colonizes better with the help of Xp. The plant's immune response raised by Pc also managed to delay the cell death susceptibility of Xp and caused delayed symptom development. In fields, when the weather is warm and humid, we can expect to see higher bacterial spot disease severity in presence of the co-occurring bacterial species compared to if the dominant pathogen was present alone. Xp makes it easier for Xa and Pc to colonize the host plant, but their presence becomes detrimental for the Xp population and reduces it. Increased growth rate of Xp in presence of Pc and Xa suggested survival attempt of Xp to maintain its population as other studies have observed that lower density of a bacterial population, in a mixture of bacterial species, might get eliminated by another microbial predator.

In case of a possible tomato bacterial spot disease outbreak during hot and humid weather, in the fields of Alabama, where we observed the co-occurrence of these three bacterial species, farmers can expect to see higher disease severity with a lower *Xanthomonas perforans* population load. Presence of the co-occurring opportunistic bacterial species in the field along with the dominant pathogen, makes the plants more susceptible to the disease progression and crop loss.

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3. CHAPTER THREE

Dissecting the interactions among Xanthomonas perforans, Xanthomonas arboricola and Pseudomonas cichorii using in vitro experiments and genome analysis

Introduction

Plant leaf phyllosphere is home for diverse microorganisms. Microbes can colonize both leaf surface and internal sites of the leaf tissue or apoplast. Among all other microbial communities like yeast, fungi and algae, bacteria are the most abundant one. In the leaf phyllosphere bacterial population, we can find epiphytes, opportunists, and saprophytes.

The leaf surface is considered as a hostile environment for microbes that try to colonize it. The survival of bacterial population on leaf surface is depended on various factors like the temperature fluctuation, solar radiation, osmotic stress etc. (Lindow & Brandl, 2003; Rastogi et al., 2013). The bacterial colonists also experience nutrient limitation and alteration between presence and absence of free moisture due to rain and dew (Wilson et al., 1999). Studies show that addition of nutrients to the leaf phyllosphere resulted in an increase in epiphytic population (Wilson & Lindow, 1994b). Limited nutritional resources can drive bacterial populations to compete. Carbon resources are relatively more limited than nitrogen resources (Wilson & Lindow, 1994b). The interaction between all these factors and how they influence microbial life is poorly understood. Most areas of leaves maintain only small amounts of nutrients. In the apoplast, bacteria encounter slight acidity and oxidative stress, which is the result of plant's defense system (Yu et al., 2013). It's poorly understood how leaf surface sites differ from the interior sites in selecting for microbial colonization traits (Yu et al., 2013).

Distinct bacterial interaction with their environment takes place at different spatial scales. The apoplastic space of leaves is a large intercellular space inside leaf tissue. (Beattie & Lindow, 1995). The phyllosphere is considered a nutrient deficient environment for microbial colonizers. Because sugars like sucrose, fructose, and glucose are present only in some particular localized sites on a leaf (Leveau & Lindow, 2001; Miller et al., 2001) and most microbes experience the nutrient poor area of leaf surface while some randomly land on a leaf area with relatively abundant nutrients (Monier & Lindow, 2005; Whipps et al., 2008). However, it has been observed that bacteria can modify the environment for better colonization. Studies show Indole-3-acetic acid (IAA) producing bacteria might aid in enhanced nutrient leakage (Brandl & Lindow, 1998). IAA-nonproducing bacterial species or opportunists present in the phyllosphere can also possibly take advantage of this enhanced nutrient leakage when a plant host is infected by multiple bacterial species or mixed infection. Phytopathogenic bacteria can also produce extracellular polysaccharide (EPS) which prevents desiccation and favors survival of the cells (Beck von Bodman & Farrand, 1995). EPS is considered analogous to biofilms where nutrient concentration can be high and more than one bacterial species can interact with each other (Beattie & Lindow, 1999). Nutritional resources have always been considered as a factor that can cause antagonism among bacterial species present on the leaf phyllosphere. Studies have revealed that in case of mixed infections in plants, one bacterial species can effectively reduce the population of another bacterial species by preemptive utilization or exhaustion of mutually required growth limiting nutritional resources (Wilson, 1992, 1993). Some foliar pathogen species are unable to compete and do not get the opportunity to acquire a sufficient portion of growth limiting resources (Wilson et al., 1989). Bacterial populations can also decline by depletion of nutrients and accumulation of toxic metabolites or by a different form of habitat modification (Wilson, 1993).

Gene expression studies of the plant pathogen *Pseudomonas syringae* revealed that the apoplastic environment can favor production of secondary metabolites like phytotoxins or compounds that might suppress plant host defense, which helps in colonization of the pathogen (Yu et al., 2013). Production of bacteriocin by plant pathogens like *Pseudomonas* spp. and *Xanthomonas* spp. has been observed in many studies (Hert et al., 2005; Los Santos et al., 2005).

Interactions between more than one microbial pathogen that co-occur in the phyllosphere has been observed and they tend to shape leaf microbial community and the colonization processes (Chaudhry et al., 2021). Studying the nutritional profile of the bacterial species that co-occur in the tomato leaf phyllosphere will allow us to gain some knowledge about the pattern of interaction between them. More about the bacterial interaction can be learned by evaluating bacteriocin production which might help the bacterial species to compete against each other. Other than characterization of these direct interactions, genome analysis of the co-occurring bacterial species can give some idea about the presence of Type III effector proteins which plays a role in making the bacteria virulent (Deng et al., 2017; Hueck, 1998; Snelders et al., 2018).

Our *in planta* mixed infection experiments with *Xanthomonas perforans*, *X. arboricola* and *Pseudomonas* species indicated that mixed infection influences the disease dynamics by affecting overall growth of dominant pathogen *X. perforans* as well as enhancing growth of opportunistic pathogens *X. arboricola* and *Pseudomonas* species. In this chapter we studied if co-occurring bacterial species can also alter the population of the dominant pathogen in absence of the "plant component". In this study we will have the opportunity to understand the interactions among the three bacterial species without the influence of host-microbe interaction.

Material Methods

Bacterial strains, media, and growth conditions

Bacterial strains used in this study are listed in Table 3.1 and the method of their construction has been described in chapter 2. These strains were grown on Nutrient agar (NA) (Difco) containing the antibiotics for the respective bacterial strains for 24 hours at 28^{o}C. All the strains were stored in 30% glycerol at -80^{o}C.

Table 3. 1: Bacterial strains used in this study

Strain	Expressed protein	Short name; Features	Source
Xanthomonas perforans AL65::Tn7-mre152	sGFP2	Xp; green fluorescent, chloramphenicol, kanamycin, and streptomycin resistant	This study
Xanthomonas arboricola CFBP 6826::Tn7-mre160	mTagBFP2	Xa; blue fluorescent, chloramphenicol, and tetracycline resistant	This study
Pseudomonas species 93B.260::Tn7-mre145	mScarlet-I	Pc; red fluorescent, chloramphenicol, and gentamicin resistant	This study

Bacterial growth curve assay

Media for the growth curve was XVM2, a minimal medium, that has been found to mimic apoplastic conditions (Jiang et al., 2013). Bacteria were cultured overnight in nutrient agar plates at 28°C. Mass of bacterial cell cultured was suspended in sterile distilled water and centrifuged down and washed and diluted to OD_{600} = 0.3 in sterile water. Next it was diluted to a final concentration of 10^6 CFU/mL for inoculation. 50 µl of the bacterial culture (10^6 CFU/ml) was inoculated in 1 ml of XVM2 media. 200 µl of inoculum for each treatment was added in the respective wells of a 96 well plate. The plate was covered and incubated at 28°C with 250 rpm shaking condition. Absorbance at 600 nm was measured every 12 hours. Fluorescence intensity was also measured at the same time points for bacterial strains tagged with fluorescent molecule producing gene in their chromosomal DNA. Xp tagged with GFP has excitation peak of 495 nm and emission peak is 512 nm. Xa tagged with BFP2 has excitation peak of 402 nm and emission peak of 457 nm. Pc tagged with mScarlet-I has excitation peak of 569 nm and emission peak of 593 nm (Schlechter et al., 2021). This experiment was repeated 4 times.

Biolog assay or determination of nutritional similarity (NOI)

Pure cultures of the bacterial strains were grown on BUG (biology universal growth) agar for 24 hours at 28°C. Three different microplates were used for three bacterial strains. From the petri plates, 3 mm diameter of cell growth was used to prepare the inoculum. The inoculum was suspended in inoculating fluid (IF-A). Cell density was adjusted to 95% transmittance. Next the biolog microplate (GEN III Microplate) was filled with 100 µl of the inoculum in each well. The plates were incubated for 48 hours at 28°C. Result was interpreted manually by observing the color

change and measuring the optical density. Nutritional similarity was calculated with the formula NOIc = the number of carbon sources used by both the nonpathogenic bacterium and the pathogen/the total number of carbon sources used by the pathogen (Wilson & Lindow, 1994a). This procedure was repeated thrice.

Bacteriocin Assay to test for in-vitro antagonism

Nutrient agar glass plates were prepared to check the bacteriocin activity of the bacterial strains. To look at the *Xanthomonas perforans* AL65::Tn7-mre152 (Xp) bacteriocin activity, a bacterial culture of *Xanthomonas perforans* AL65::Tn7-mre152 (Xp) was prepared with a final concentration of 10^8 CFU/ml. 10µl of this bacterial culture was used as inoculum, and it was drop spotted in the center of the nutrient agar plate. The plates were incubated for 48 hours at 28°C. After incubation, the plates were inverted, and 2 to 3 ml of chloroform was added to the lid, and it was allowed to completely dry for several hours. Next, the plates were aerated inside biosafety cabinet for an hour. Next, overnight grown bacterial cultures of *Xanthomonas arboricola* CFBP 6826 (Xa) & *Pseudomonas* species 93B.260 (Pc) were suspended separately in $MgSO_4$ buffer and centrifuged down and washed and diluted to OD_{600} = 0.3 in $MgSO_4$ buffer solution. Two different solutions of Xa and Pc were prepared by using 200µl (OD_{600} = 0.3) of each bacterial strain as inoculum, to add in 3.5ml of 0.3% water agar. Water agar solution of Xa and Pc were vortexed thoroughly and overlayed on top of two different aerated plates to see the bacteriocin activity of Xp against Xa and Pc. The plates were secured with parafilm and incubated for 48 hours. This method was repeated to also observe the bacteriocin activity of *Xanthomonas arboricola* CFBP 6826 (Xa) & *Pseudomonas* species 93B.260 (Pc) against the other bacterial strains. The assay was repeated twice.

Contact dependent inhibition assay

The bacterial strains were grown on nutrient agar plates at 28°C for 24 hours. Nutrient agar plates and NYG plates were prepared for the assay. A loop full of bacterial inoculum was used from each strain to inoculate a circular area of 0.7 mm diameter. Bacterial strains were streaked next to each other in combination of two or three. Plates were allowed to grow for 2-3 days at 28°C and checked for result. Presence of Crescent shaped colony was considered as an indication of contact dependent inhibition. The influence of the weak bacterial strains (*Xanthomonas arboricola* CFBP 6826::Tn7-mre160 (Xa) & *Pseudomonas* species 93B.260::Tn7-mre145 (Pc)) were checked against the dominant bacterial pathogen *Xanthomonas perforans* AL65::Tn7-mre152 (Xp). The experiment was repeated twice.

Skimmed milk agar assay to observe protease activity

Skimmed milk agar assay was conducted to detect the presence of extracellular cell bound protease in *Xanthomonas perforans* AL65::Tn7-mre152 (Xp), *Xanthomonas arboricola* CFBP 6826::Tn7-mre160 (Xa) & *Pseudomonas* species 93B.260::Tn7-mre145 (Pc). Skimmed milk agar plates were prepared in the following way: 25 grams of nonfat dry milk was reconstituted in 250 ml of distilled water. Next the mixture was stirred thoroughly and autoclaved at 121°C for 15 minutes. 500 ml of 2·5% agar solution was also sterilized. Sterilized skimmed milk and 2.5% agar solution were mixed maintaining aseptic condition. The skim milk agar solution was poured quickly into plates. Skimmed milk agar plates were inoculated with loop full of bacterial culture of each strain. Inoculated plates were incubated at 28°C for 5 days. Protein digestion or presence of proteases was detected by the production of clear haloes surrounding the colonies (Pailin et al., 2001).

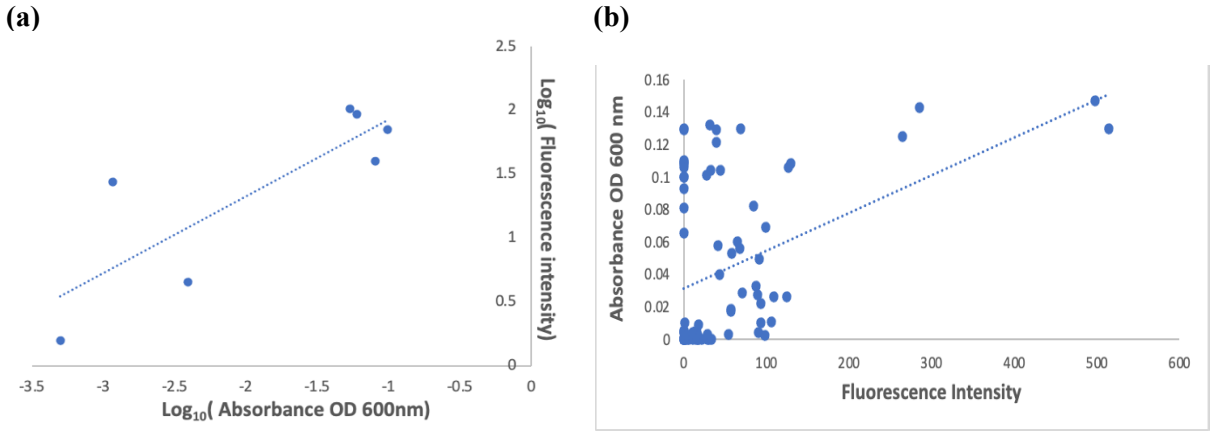
Genome analysis to understand functional potential of organisms

Searches for plant cell wall degrading enzyme COGs (cluster of orthologous groups) were performed using https://img.jgi.doe.gov in *Xanthomonas arboricola* CFBP 6826 genome and *Pseudomonas* species 93B.260 genome. BLAST was performed with the whole genome of *Xanthomonas arboricola* CFBP 6826 against T3SS genes and Type 3 effector protein database. Genome of *Pseudomonas* species 93B.260 was also blasted against a database of Type 3 effectors produced by plant pathogenic *Pseudomonas syringae* pv. tomato DC300 strain. *Xanthomonas* sp. database for these secretion systems and effectors was created from *Xanthomonas euvesicatoria* and *Xanthomonas* campestris pv. vesicatoria 85-10. Percent identity more than or equal to 40% with a query coverage of 30% was considered as a similar product.

Data analysis

Growth rate, generation time or doubling time and carrying capacity of each bacterial strain was calculated using growthcurver (Sprouffske & Wagner, 2016) package of R. Data analysis and visualization was done using GlIMMIX procedure in SAS. Analysis of variance (ANOVA) repeated measurements were performed to evaluate significant difference among the growth pattern of various treatments. All data used for ANOVA were normally distributed. Pearson's correlations between fluorescence intensity and absorbance were determined using SAS.

Results



Correlation between Log of Absorbance of Xp & Log of Fluorescence intensity of Xp

Correlation between Absorbance of Xp & Fluorescence intensity of Xp

Figure 3. 1: Positive correlation between Xp absorbance and Xp fluorescence intensity.

Simple linear regression model of (a) Log of Xp fluorescence intensity versus Log of Xp absorbance & (b) Xp fluorescence intensity versus Xp absorbance

Table 3. 2: Parameters derived from linear regression models and correlation between the fluorescence and absorbance measurements for Xp presented in Figure 3.1(a) & (b)

Figure	**Figure 1a**	**Figure 1b**
Response variable	**Log_{10} (Fluorescence intensity of Xp)**	**Optical density 600 nm of Xp**
Explanatory variable	**Log_{10} (Optical density 600 nm of Xp)**	**Fluorescence Intensity of Xp**
R^2	**0.6857**	**0.17263278**
Adjusted R^2	**0.62289993**	**0.16254294**
Pearson's correlation	**0.8281002**	**0.41549102**
P value	**0.02**	**0.0000848705**

Fluorescence intensity and optical density of Xp has a positive correlation

Study done by Schlechter et al., 2021 used *Pantoea eucalypti* 299R::Tn7-mre145, made by using the construct pMRE-Tn7-145, which has also been used in our study to make *Pseudomonas cichorii* 93B.260::Tn7-mre145, for constitutive expression of mScarlet-I (red fluorescent protein). They also used different constitutively expressed fluorescence proteins to measure bacterial growth by tagging different bacterial strains with similar plasmid constructs (pMRE-Tn5-143, pMRE-Tn5-145) by Schlechter et al., 2018 (Schlechter et al., 2018). They used simple linear regression models to analyze the correlation between different measures of the optical density and fluorescence data. The results of their study indicated, increase in fluorescence in a population could be associated with an increase in the population density instead of an increase in the protein maturation rate. Statistically significant and positive correlation was observed between the fluorescence intensity curves of bacterial strains and the final $OD_{600.}$ It was concluded that fluorescence signals can be used to track the bacterial growth in liquid cultures, because fluorescence can be used as an alternative to OD (Schlechter et al., 2021). In our study we also tested if constitutively expressed sGFP by Xp could be used as a proxy for bacterial growth or not. We plotted simple linear regression model by using the log of optical density of pure Xp bacterial culture measured at 600 nm and log of fluorescence intensity of Xp, after they were excited at 495 nm and the emission was measured at 512 nm (Figure 3.1a). In figure 3.1a the log of optical density of Xp and log of fluorescence intensity of Xp had a positive correlation with a Pearson's correlation value of 0.8281002, but it was not significant. We also plotted another simple linear regression model by using the optical density of pure Xp bacterial culture measured at 600 nm and fluorescence intensity of Xp, after they were excited at 495 nm and the emission was measured at 512 nm (Figure 3.1b). In this linear regression model the adjusted R^2 was 0.16254294 and

Pearson's correlation was 0.41549102 which indicates a weakly positive correlation between fluorescence intensity and optical density of Xp which was significant ($P<0.0001$). Therefore, we can use fluorescence intensity of Xp as an indicator of its bacterial growth for this study.

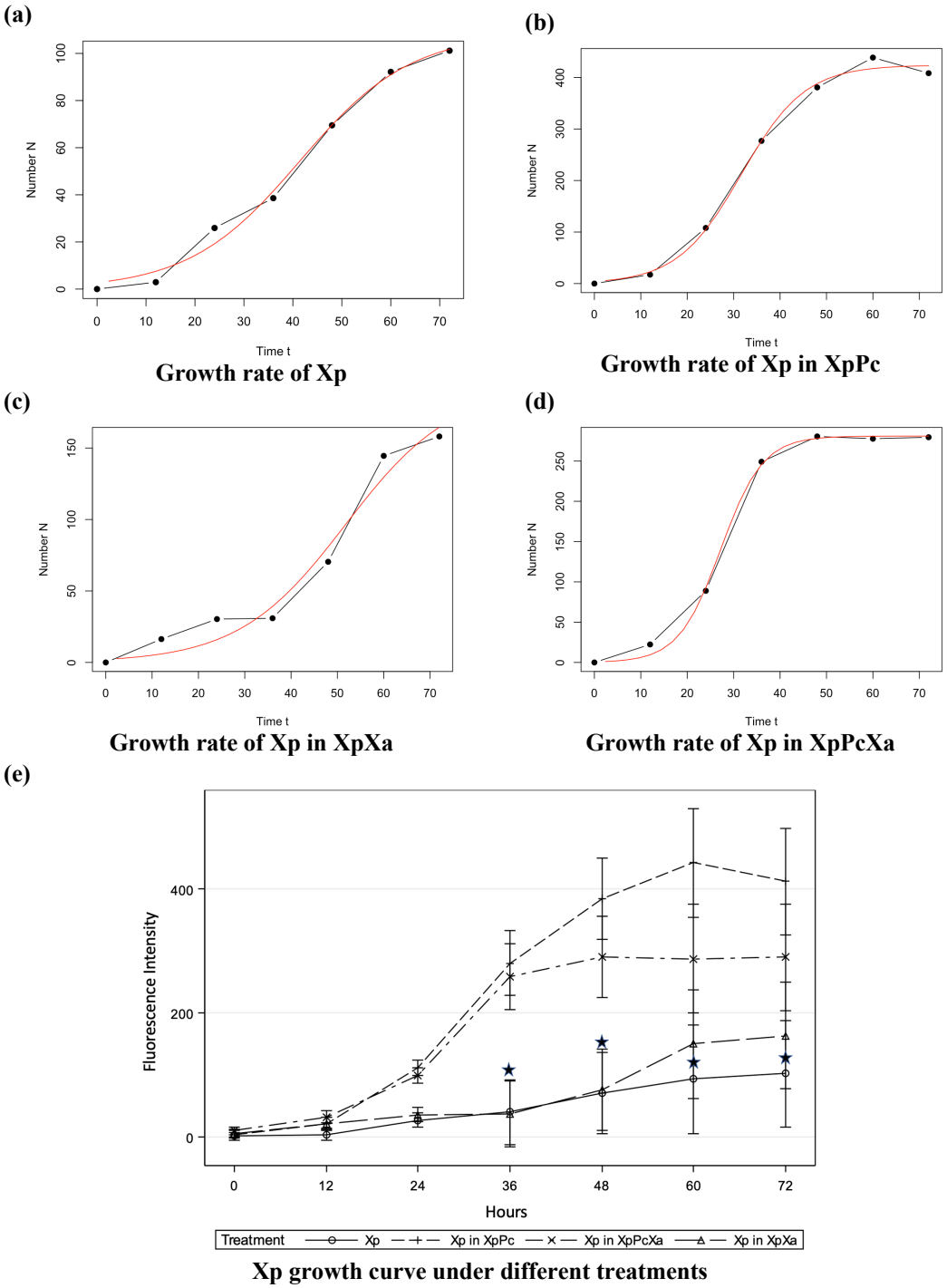


Figure 3. 2: Xp Growth rate and Xp population measured by fluorescence intensity in pure and mixed culture. Xp growth rate (a) in Xp pure culture (b) in XpPc (c) XpXa and (d) XpPcXa mixed cultures. (e) In-vitro Xp population growth in absence and presence of Pc and Xa. Vertical

lines represent the 95% confidence limit. Treatment Xp with * marks have significantly lower population compared to population of Xp in XpPcXa and XpPc according to Tukey's test of least significant difference ($P<0.05$). To measure the fluorescence intensity of GFP tagged Xp, the cells were excited at 495 nm and emission was measured at 512 nm.

Table 3. 3: Growth rate of Xp under different treatments presented in Figure 3.2

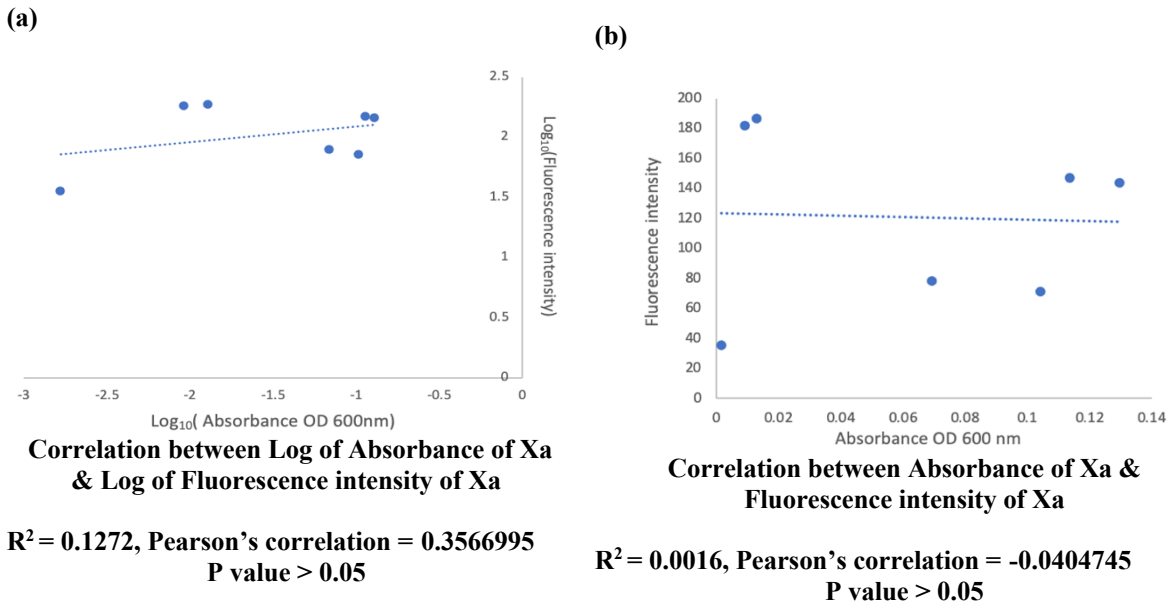
Treatment	Growth rate (h^{-1})	Doubling time (hours)	Carrying capacity (Fluorescence intensity)
Xp	0.08832592	7.85	109
Xp in XpPc	0.1458811	4.75	424
Xp in XpXa	0.08671989	7.99	193
Xp in XpPcXa	0.2208645	3.14	281

Xp exhibits lowest population and carrying capacity in absence of Pc & Xa, but Xp growth rate is highest in presence of Xa and Pc

Our goal was to understand the inter-species relationships among Xp, Pc and Xa in a one-on-one fashion. Since we observed a positive correlation between fluorescence intensity and optical density of Xp, we measured the fluorescence intensity of Xp as an indicator of its growth, in pure culture and in presence of mixed cultures containing the co-occurring bacterial species (Xp + Pc / Xp +Xa / Xp + Xa + Pc). Xp population was observed to be lowest when its only by itself (Figure 3.2e). For the first 24 hours there was no statistically significant increase among the treatments. At 36th and 48th hour, the fluorescence intensity of Xp alone was significantly ($p<0.05$) lower than the fluorescence intensity of Xp in the treatment XpPcXa and XpPc. In mixed cultures with Pc,

growth of Xp was significantly higher ($p<0.05$) after 24 hours. From fluorescence intensity of ~ 100 a.u. (arbitrary units), it increases up to ~ 400 a.u. (arbitrary units) within next 36 hours. Afterwards, the fluorescence intensity decreased slightly. Growth of Xp also showed significant increase ($p<0.05$) after 24 hours, when it was present in a mixed culture with both Pc and Xa. Xa alone did not seem to have the similar effect on growth of Xp. In a mixed culture with only Xa, there was no significant increase in the growth of Xp. In presence of Pc, population of Xp always had a relatively shorter lag phase and entered the log phase early with sharp increase in the population.

To understand if Pc or Xa has any influence on the carrying capacity and Xp growth rate, we measured the growth rate, doubling time and carrying capacity for Xp from fluorescence intensity observation of pure Xp bacterial culture and mixed bacterial cultures (Figure 3.2 and Table 3.3). We observed carrying capacity for Xp was lowest when it was alone. Growth rate and doubling time of Xp remained almost same, but there was a slight increase in the Xp carrying capacity, in presence of Xa as compared to growth rate of Xp in pure culture. In presence of Pc, Xp had a reduced doubling time, Xp growth rate increased from 0.08832592 h^{-1} to 0.1458811 h^{-1} and the carrying capacity for Xp in the XVM2 medium reached highest. However, Xp growth rate was highest (0.2208645 h^{-1}) with the smallest doubling or generation time of 3.14 hours, in combined presence of Xa and Pc. Joint presence of Pc and Xa, also increased the carrying capacity for Xp. Our overall observations indicate the presence of Pc increased the Xp growth rate, carrying capacity and population compared to when Xp is present only by itself.



Correlation between Log of Absorbance of Xa & Log of Fluorescence intensity of Xa

R^2 = 0.1272, Pearson's correlation = 0.3566995
P value > 0.05

Correlation between Absorbance of Xa & Fluorescence intensity of Xa

R^2 = 0.0016, Pearson's correlation = -0.0404745
P value > 0.05

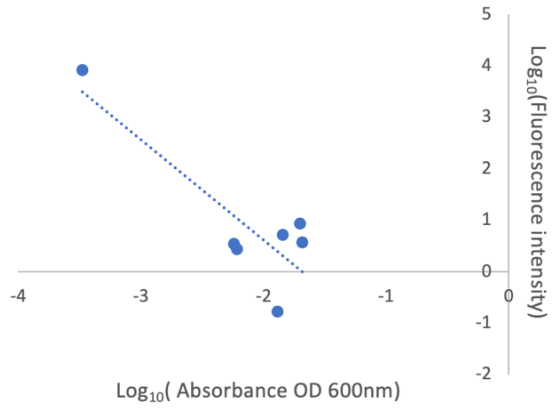
Figure 3. 3: Correlation between Xa absorbance and Xa fluorescent intensity. Xa absorbance was measured at OD_{600nm}. To measure the fluorescence intensity of BFP2 tagged Xa, the cells were excited at 402 nm and emission was measured at 457 nm. Simple linear regression model of (a) Log of Xa fluorescence intensity versus Log of Xa absorbance & (b) Xa fluorescence intensity versus Xa absorbance

Xa fluorescence intensity and Xa absorbance has a negative correlation

To examine if constitutively expressed BFP2 produced by Xa could be used as an estimate for bacterial growth we used the absorbance of pure Xa bacterial culture and fluorescence intensity of Xa to plot a simple linear regression model. To measure the fluorescence intensity, Xa bacterial culture was excited at 402 nm and the emission was measured at 457 nm. Absorbance and fluorescence intensity was measured on hour 0, 12, 24, 36, 48, 60, 72. In the linear regression model for Xa (Figure 3.3a), Pearson's correlation was -0.0404745 which indicates a negative correlation between Xa fluorescence intensity and Xa optical density with a non-significant p value (P= 0.754803748). We also plotted another simple linear regression model by using the log of

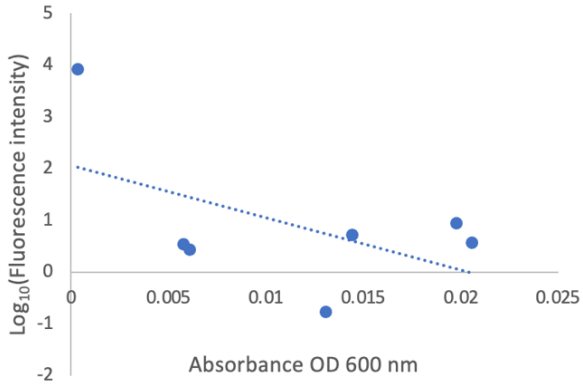
optical density of pure Xa bacterial culture measured at 600 nm and log of fluorescence intensity of Xa (Figure 3.3b) and a low positive correlation was observed with a Pearson's correlation of 0.3566995, but it was not significant (P value >0.05). As a result, the fluorescence intensity of Xa cannot be used as an indicator of the bacterial growth of Xa. Growth rate of Xa was measured 0.1770384 h^{-1} from Xa absorbance values and Xa growth rate was lower than Xp growth rate 0.5706992 h^{-1}. A possible reason for not observing positive correlation between fluorescence intensity and absorbance can be contamination with other bacterial species in the media.

(a)



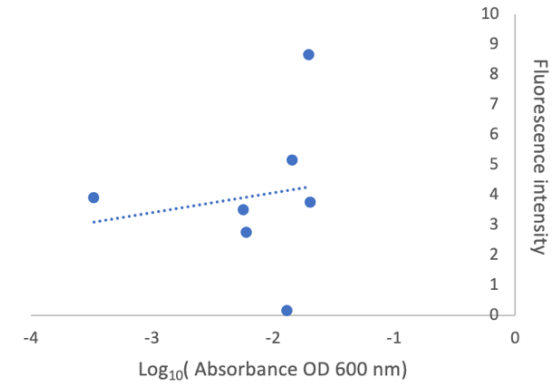
$R^2 = 0.719$, Pearson's correlation = -0.847955

(b)



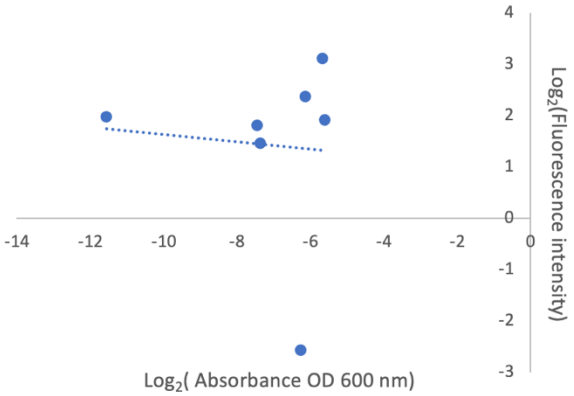
$R^2 = 0.2833$, Pearson's correlation = -0.532230257

(c)



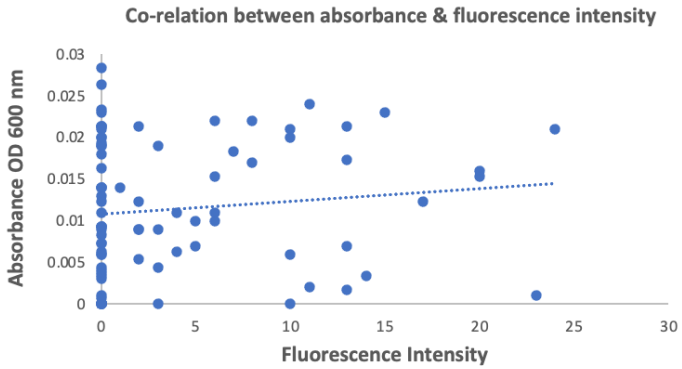
$R^2 = 0.0254$, Pearson's correlation = 0.15922878

(d)



$R^2 = 0.0065$, Pearson's correlation = -0.0807412

(e)



$R^2 = 0.01341972$, Pearson's correlation = 0.11584, P value > 0.05

Figure 3. 4: Correlation between Absorbance of Pc & Fluorescence intensity of Pc. Pc absorbance was measured at OD_{600nm}. To measure the fluorescence intensity of mscarlet tagged Pc, the cells

were excited at 569 nm and emission was measured at 593 nm. Simple linear regression model of (a) Log_{10} of Pc fluorescence intensity versus Log_{10} of Pc absorbance & (b) Log_{10} of Pc fluorescence intensity versus Pc absorbance (d) Pc fluorescence intensity versus Log_{10} of Pc absorbance (d) Log_2 of Pc fluorescence intensity versus Log_2 of Pc absorbance (e) Pc fluorescence intensity versus Pc absorbance

Correlation does not exist between fluorescence intensity and absorbance of Pc

To find correlation between fluorescence intensity and absorbance ($OD_{600\ nm}$) of Pc a simple linear regression model was plotted (Figure 3.4e). Pc bacterial culture was excited at 569 nm and the emission was measured at 593 nm to measure the fluorescence intensity. However, in the linear regression model for Pc, the adjusted R^2 was 0.00138825 and Pearson's correlation was 0.11584, which indicated no linear relationship or no correlation between Pc fluorescence intensity and Pc absorbance (p value = 0.2940). We also plotted simple linear regression model of (3.4a) Log_{10} of Pc fluorescence intensity versus Log_{10} of Pc absorbance & (3.4b) Log_{10} of Pc fluorescence intensity versus Pc absorbance (3.4c) Pc fluorescence intensity versus Log_{10} of Pc absorbance (3.4d) Log_2 of Pc fluorescence intensity versus Log_2 of Pc absorbance. None of them exhibited any significant positive correlation between fluorescence intensity and absorbance. Therefore, we cannot use fluorescence intensity of Pc as a predictor or proxy for bacterial growth of Pc. Using the 600 nm absorbance data, growth rate of Pc was determined to be 0.073875 h^{-1}, which is lower than growth rate of both Xa and Xp. Contamination with other bacterial species could have been a reason for not observing positive correlation between Pc fluorescence intensity and Pc absorbance.

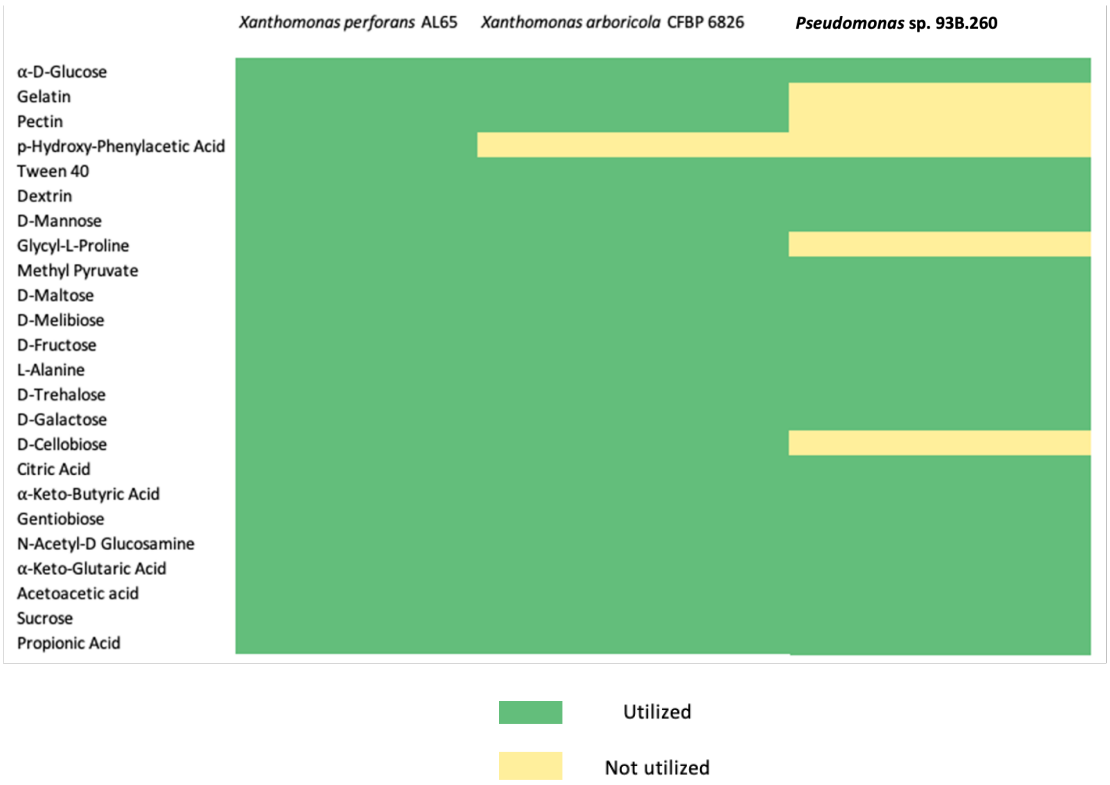


Figure 3. 5. Carbon source utilization Profile of *Xanthomonas perforans* AL65, *Xanthomonas arboricola* CFBP 6826, *Pseudomonas* species 93B.260

Table 3. 4. Nutritional similarities among the 3 different species

Bacterial Strain	**NOIc (Nutritional Similarity in Carbon source utilization)**
Xanthomonas perforans AL65 & *Xanthomonas arboricola* CFBP 6826	0.96
Xanthomonas perforans AL65 & *Pseudomonas* species 93B.260	0.81
Pseudomonas species 93B.260 & *Xanthomonas arboricola* CFBP 6826	0.84

Determination of nutritional similarity

NOI_C of *X. perforans* AL65 & *X. arboricola* CFBP 6826 = the number of carbon sources used by both *Xanthomonas arboricola* CFBP 6826 & *Xanthomonas perforans* AL65/the number of carbon sources used by the pathogen *Xanthomonas perforans* AL65

NOI_C of *X. perforans* AL65 & *Pseudomonas* species 93B.260 = the number of carbon sources used by both *Xanthomonas perforans* AL65 & *Pseudomonas* species 93B.260/the number of carbon sources used by the pathogen *Xanthomonas perforans* AL65

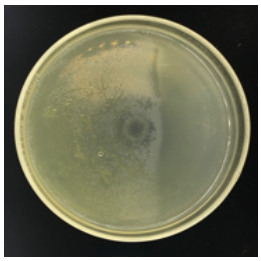
NOI_C of *X. arboricola* CFBP 6826 & *Pseudomonas* species 93B.260 = the number of carbon sources used by both *Xanthomonas arboricola* CFBP 6826 & *Pseudomonas* species 93B.260/the number of carbon sources used by *Xanthomonas arboricola* CFBP 6826

Higher nutritional similarity (NOI_C) between Xp and Xa

Our in vitro growth curve assay results suggested that presence of Pc increased the Xp population and growth rate. We also observed that presence of Xa did not cause any significant change in the Xp population or the growth rate. In chapter 2, the in-planta population assay exhibited reduced Xp population in presence of Xa and Pc compared to when plants were inoculated with Xp alone. To understand if the altered Xp population and growth rate in presence of the co-occurring bacterial species, was an outcome of the ability of the 3 bacterial species to compete for nutritional resources, we compared the nutritional profile. In vitro carbon source utilization profiles were determined for *X. perforans* AL65, *X. arboricola* CFBP 6826 and *Pseudomonas* species 93B.260 (Figure 3.5 & Table 3.4). Using the carbon source utilization profile, nutritional similarity between the strains were calculated according to Wilson and Lindow (Wilson & Lindow, 1994b).

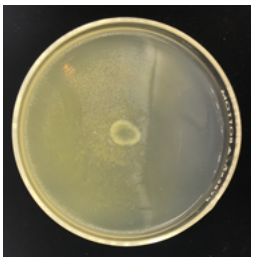
Nutritional similarity or NOI primarily focuses on the overlap in nutrient utilization between the non-pathogenic agent and the pathogen. Carbon utilization profile between strains were created by this formula NOIc = the number of carbon sources used by both the nonpathogenic bacterium and the pathogen/the total number of carbon sources used by the pathogen. The highest NOI value is 1.0, and it indicates higher nutritional similarity between the two bacterial species. The carbon utilization profile between *X. perforans* AL65 & *X. arboricola* CFBP 6826 is 0.96, which is the higher than the others. Niche overlap values greater than 0·9 is indicative of utilization of the same nutrients (Lee & Magan, 1999; Wilson & Lindow, 1994a). The NOIc between *X. arboricola* CFBP 6826 & *Pseudomonas* species 93B.260 is 0.84 and the NOIc between *X. perforans* AL65 & *Pseudomonas* species 93B.260 is 0.81. The results indicated that there is more nutritional overlap among *X. arboricola* CFBP 6826 and *X. perforans* AL65 but lower among *Pseudomonas* species 93B.260 and *X. perforans* AL65. Presence of Pc increased the Xp population and growth rate possibly due to lesser nutritional similarity between Xp and Pc compared to nutritional similarity between Xp & Xa.

(a)



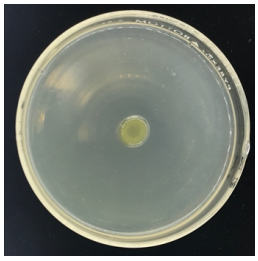
Test strain: Pc against Indicator strain: Xp
Inhibition Zone: Absent

(b)



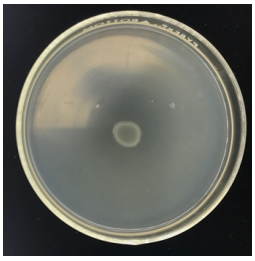
Test Strain: Xa against Indicator strain: Xp
Inhibition Zone: Absent

(c)



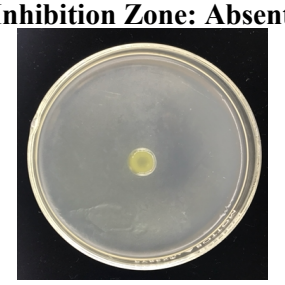
Test strain: Xp against Indicator strain: Xa
Inhibition Zone: Absent

(d)



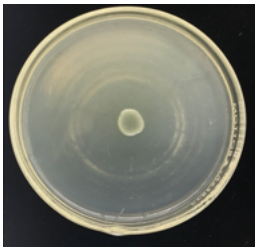
Test strain: Pc against Indicator strain: Xa
Inhibition Zone: Absent

(e)



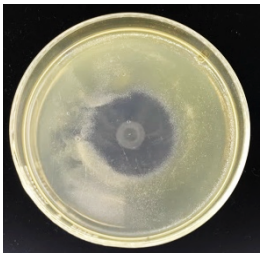
Test strain: Xp against Indicator strain: Pc
Inhibition Zone: Absent

(f)



Test strain: Xa against Indicator strain: Pc
Inhibition Zone: Absent

(g)



(Positive Control) Test strain: *Enterobacter cloace* against Indicator strain: Xp
Inhibition Zone: Present

Figure 3. 6: Bacteriocin Assay for detection of in-vitro antagonism. Bacteriocin like activity was tested by drop spotting the test strain in the middle of agar plate and using 0.3% water agar solution containing 200µl (OD_{600} = 0.3) of indicator strain inoculum, as overlay. Zone of inhibition/clearance or halo is the indicator of bacteriocin like activity against indicator strain.

Xp, Pc and Xa did not produce bacteriocin against each other

Nutritional similarity among the bacterial species indicated niche overlap and possible competition among them. To detect if production of bacteriocin was also used by any of these bacterial species as a mechanism for competing, bacteriocin production assay was performed. Bacteriocins are antimicrobial compounds, which are proteinaceous in nature and produced by bacteria. Bacteriocins are usually narrow spectrum and harmful towards closely related bacteria. (Holtsmark et al., 2008). Production of halo zone around the test strain in the middle of the plates were considered as an indicator of bacteriocin activity. *Enterobacter cloace* was used as a test strain against Xp for positive control. Clear halo zone was observed around *Enterobacter cloace* in the middle of the plate, which indicated production of bacteriocin or bacteriocin like compounds (Figure 3.6g). We checked if Pc and Xa had any bacteriocin like activity against Xp (Figure 3.6a & b). No clear zone of inhibition or halo was observed which indicates that Pc or Xa do not have the capability to inhibit the growth of Xp by production of bacteriocin or bacteriocin like compounds. Similarly, we evaluated the bacteriocin production potential of Xp and Pc against Xa (Figure 3.6c & d). No zone of inhibition was produced by either of them. We also checked if Xp and Xa can produce any bacteriocin or bacteriocin like compound to inhibit Pc (Figure 3.6e & f). No bacteriocin like activity was detected as there was no clear zone or halo was observed. The experiment was repeated twice, and same results were observed both times. Therefore, we can eliminate the possibility of competition among these 3 bacterial species by production of bacteriocin. However, if the nature of the interaction among Xp, Pc and Xa is competitive, they might be competing via other mode of actions like competition for resources or space in the leaf phyllosphere.

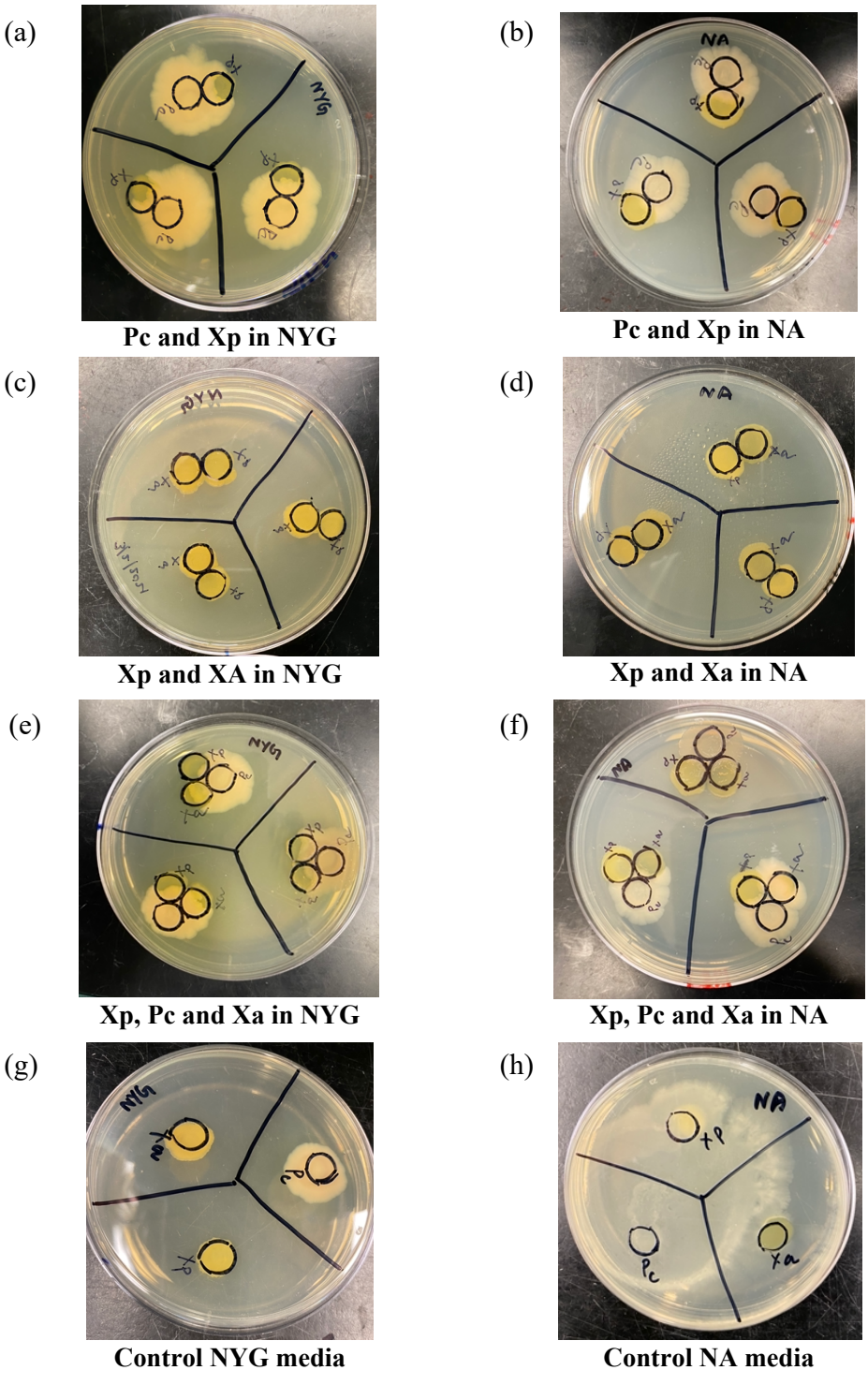
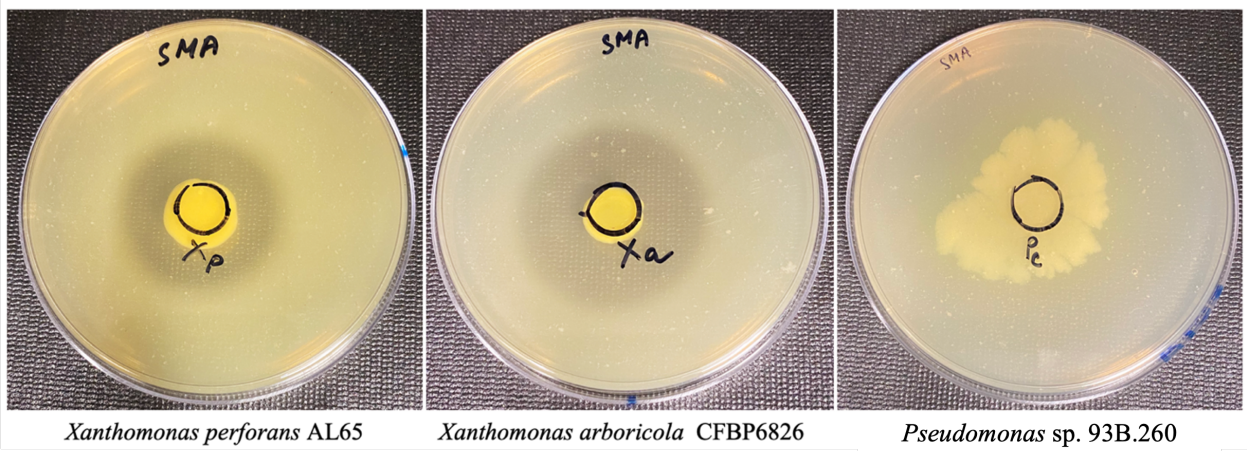


Figure 3. 7: Contact dependent inhibition among Xp, Xa and Pc. A loop full of bacterial inoculum were used for each strain to streak inside a circular area of 0.7 mm diameter. Bacterial

strains were streaked next to each other in combination of two or three. Development of Crescent shaped colony was considered as an indication of contact dependent inhibition. No inhibition was observed.

No contact dependent inhibition was observed among Xp, Pc and Xa.

Xp and Pc did not inhibit each other's growth when their colonies were placed in proximity (Figure 3.7a & b). In both NYG (nutrient yeast glycerol) agar media and nutrient agar media, overgrowth of the Pc colony was observed but it did not inhibit the growth of Xp. Xp and Xa did not inhibit each other's growth in nutrient agar media (Figure 3.7c & d). We observed the same thing in NYG media. None of them overgrew each other. When all three of them, Xp, Pc and Xa were inoculated next to each other in NYG and Na media (Figure 3.7e & f), we observed the same growth pattern of Pc. Pc grew more than Xp and Xa. However, none of the strains inhibited each other. Aa a positive control all three strains were inoculated separately in NA and NYG plates (Figure 3.7g & h). We observed that Pc grew more than Xa and Xp in NYG. However, in NA control plates the growth of Pc was visibly than its growth in NYG media, which almost covered half of the plate with. Previously we calculated the growth rate of Pc in XVM2 media, which was slower compared to Xp and Xa. XVM2 media is a minimal media, and it mimics plant apoplast environment. The results indicates that Pc grows poorly in minimal media compared to Xp and Xa but grows more than Xa and Xp in a basal media like nutrient agar where enough nutrients are present.



Xanthomonas perforans AL65 *Xanthomonas arboricola* CFBP6826 *Pseudomonas* sp. 93B.260

Figure 3. 8: Protease activity of Xp, Xa & Pc. Protease activity was tested by inoculating a loop full of bacterial culture in the middle of the skimmed milk agar plate. Zone of clearance or halo is indicator of the protease activity.

Xa and Xp exhibit protease activity.

Detection of protease production by bacterial pathogen is a prerequisite for understanding their role in the pathogenesis of infectious diseases. Qualitative determination of protease activity is done by Skimmed milk agar plate assay. Use of lactose-free and fat-free skimmed milk powder as the substrate for protease activity detection, allows discrimination against acid production because lactose hydrolysis will not be an issue. Clear zones were determined as indicator proteolytic activity (L. S. Morris et al., 2012). Figure 3.8 shows presence of zone of clearance produced by Xa and Xp in skimmed milk agar plates after 4-5 days. Plates inoculated with Pc did not have any zone of clearance. It indicates Pc lacks the principal protease enzymes. Lack of principal protease enzymes makes Pc a poor candidate for colonizing host plants.

Table 3. 5: Presence of cell wall degrading enzymes in Xa and Pc. COG (Clusters of Orthologous Groups)

Strain	COG name	COG ID	Presence
Xanthomonas arboricola CFBP 6826	Pectate Lyase (pectinase)	COG3866	Yes
	Cellulase or cellobiose CelA1	COG5297	No
	Copper oxidase laccase (ligninase)	COG1496	Yes
	Endo-glucanase (degrades cellulose)	COG3405	Yes
	Beta-xylosidase (hemicellulase)	COG3507	Yes
	Endo-1,4-beta-xylanase (hemicellulase)	COG3693	yes
Pseudomonas sp. 93B.260	Pectate Lyase (pectinase)	COG3866	No
	Cellulase or cellobiose CelA1	COG5297	No
	Copper oxidase laccase (ligninase)	COG1496	No
	Endo-glucanase (degrades cellulose)	COG3405	No
	Beta-xylosidase (hemicellulase)	COG3507	No
	Endo-1,4-beta-xylanase (hemicellulase)	COG3693	No

Principal cell wall degrading enzymes are absent in Pc

Plant cell wall is mad of cellulose, lignin, hemicellulose, and pectin. Microbial pathogens possess cell wall degrading enzymes which can cause plant cell wall degradation and macerate plant tissue (Marin-Rodriguez, 2002). Bacterial cellulase can also degrade plant cell wall and releases the cell contents (Sadhu, 2013). These two enzymes help plant pathogens to overcome the physical barrier to establish a successful infection. Presence and absence of primary cell wall degrading enzymes were evaluated in *Xanthomonas arboricola* CFBP 6826 and *Pseudomonas* species 93B.260 by finding COGs (cluster of orthologous groups) using https://img.jgi.doe.gov (Table 3.5). COG analysis uses evolutionary relations (orthologs) to group functionally related gene. Results indicated *Xanthomonas arboricola* CFBP 6826 has most of the cell wall degrading enzymes like pectate lyase (pectinase), endo-glucanase (degrades cellulose), endo-1,4-beta-xylanase (degrades hemicellulose), beta-xylosidase (degrades hemicellulose), copper oxidase laccase (degrades lignin). However, none of these enzyme producing genes are present in *Pseudomonas* species 93B.260, which indicates that *Pseudomonas* species 93B.260 is possibly a poor colonizer of its host plant.

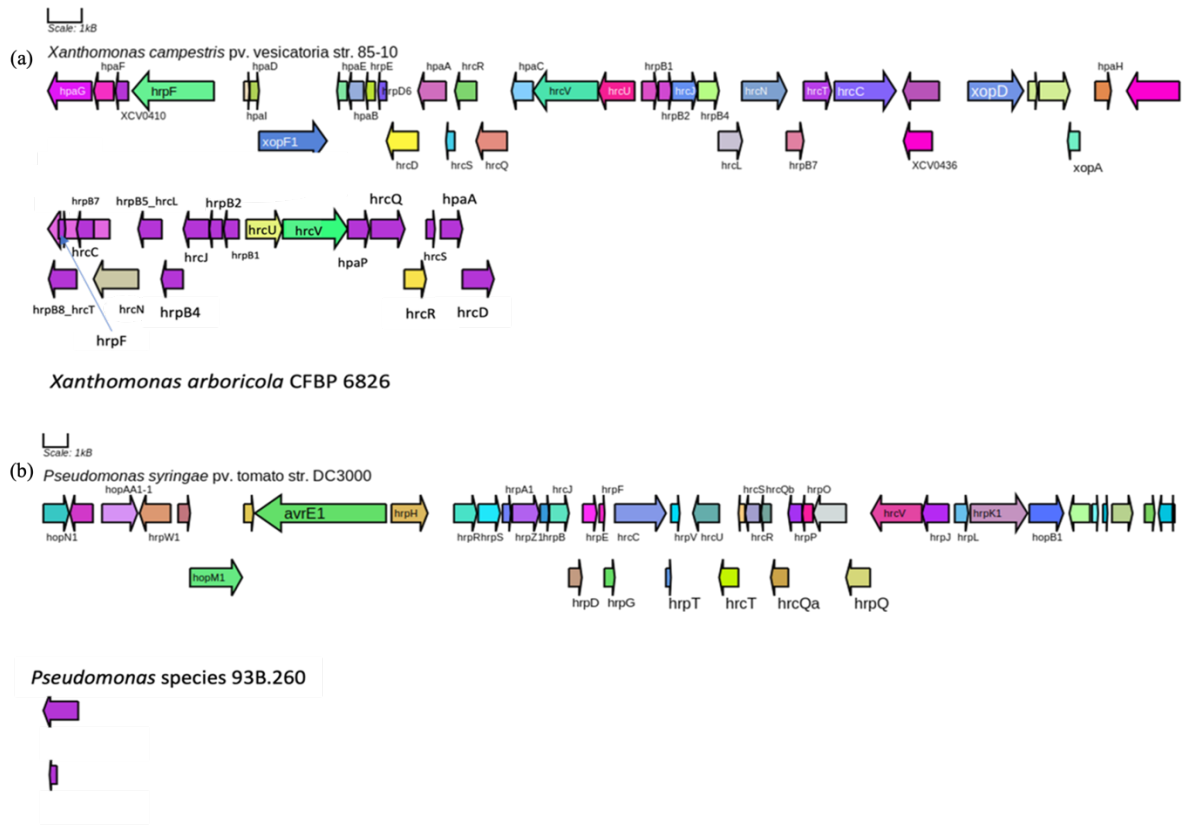


Figure 3. 9: Variation in T3SS cluster organization of *X. arboricola* CFBP 6826 and *Pseudomonas* species 93B.260. Genes are represented by colored blocked arrows. (a) Comparison between *X. campestris* pv. vesicatoria strain 85-10 and *X. arboricola* CFBP 6826. (b) Comparison between *P. syringae* pv tomato strain DC300 and *Pseudomonas* species 93B.260

Table 3. 6: Type III effectors in *X. arboricola* CFBP 6826 and *Pseudomonas* sp. 93B.260

***Xanthomonas arboricola* CFBP 6826**						
Effector	**Percent identity**	**Mismatches**	**Gap openings**	**Query Coverage %**	**E(expect)-value**	**bit score**
AvrBs2_Xeu	76.40	165	3	99.72	0	997
XopZ_Xg	97.00	39	0	98.94	0	2021
XopAL1_Xcc	99.10	3	0	100	0	640
XopAW_Xcc	95.24	8	1	85.07	4.59E-40	143
HrpW_Xeu	81.85	35	1	76.23	4.47E-136	424
XpF_Xeu	69.24	203	2	99.85	3.51E-06	48.1
XopR_Xoo	44.80	85	0	35.24	1.32E-36	140
XopAD_Xp	65.64	574	4	62.09	0	1897
XopAR_Xp	55.97	57	1	100	2.32E-33	120
HpaA_Xeu	58.39	110	1	99.64	6.14E-47	165
***Pseudomonas* sp. 93B.260**						
hopBD2	76.923	48	1	88.26	1.24E-132	412
avrE1	42.75	829	19	91.3	0	1202
hopBD1	52.8	96	2	70.33	9.58E-72	238
hopBJ1	49.24	128	4	94.18	1.95E-70	233
hopBN	67.22	94	2	99.66	1.24E-132	412

Xa has T3SS and produces Type III effector proteins

Relatively higher similarity was observed in nutritional profiles of Xp and Xa which indicated possible competition for resources. To understand if it's possible for Xa to become a virulent pathogen with help of its Type III secretion system (T3SS), we analyzed the Xa T3SS cluster organization and Type III effector production. T3SS genes and effectors are believed to play role in pathogenicity, virulent functions and host defense (Potnis et al., 2011). T3SS cluster of *X. campestris* pv. vesicatoria 85-10 was compared with Xa. Presence of a type III secretion system with *hrp* conserved (*hrc*) genes were detected in Xa (Figure 3.9a). Type III effectors like AvrBs2, XopZ, XopAL1, XopAW, HrpW, XopAR, XopAD, XpF are also detected in *Xanthomonas arboricola* CFBP 6826 (Table 3.6). Xa can produce relatively higher number of effectors compared to Pc and can possibly cause more damage to the host plant than Pc.

Absence of a complete Type III Secretion System in Pc

We observed that the primary host cell wall degrading enzymes are absent in Pc, also it could not produce protease. To further confirm the weak nature of Pc we investigated it's Type III secretion system and effectors since they confer virulence to plant pathogenic bacteria. Comparison of Type III secretion system gene clusters between virulent strain Pseudomonas syringae pv. tomato DC300 and *Pseudomonas* species 93B.260 showed that *Pseudomonas* sp. 93B.260 lacks the T3SS genes, and two hypothetical protein producing genes were detected to be similar after running blast (Figure 3.9b). Effector hopBD2, hopBD1, avrE1, hopBJ1 and hopBN was found after blasting against Type III secretion system effectors (Table 3.6). Absence of the T3SS genes and relatively a lesser number of effectors indicate that Pc is a weak pathogen.

Discussion

In this chapter we tried to study the nature of interaction among the three co-occurring bacterial species Xp, Xa and Pc, without the involvement of the plant host by conducting in vitro experiments. In chapter 2 we observed higher disease severity in mixed infected plants co-inoculated with Xp and Pc or Xa, with significantly lower Xp population compared to Xp single infection. To understand the reason behind lower Xp population in mixed infections, we tried to understand if the bacterial species compete against each other or not. No bacteriocin production was detected by any of the bacterial species as a mode of competition, neither did we observe any contact dependent inhibition among the three bacterial species. Genome analysis data revealed that Type III secretion system genes and Type III effectors were absent in Pc. Xa can produce different Type III effectors and also have the T3SS cluster. We also assessed the nutritional profile of the three bacterial species which indicated higher nutritional similarity between Xp and Xa and possible competition for nutrients. We also enumerated the Xp population from mixed inoculations under in-vitro conditions and we detected lowest population of Xp when Xp was inoculated alone, opposed to our in-planta observation where Xp population was highest when plants were inoculated with only Xp. However, the growth rate of Xp population was observed to be the highest in presence of the two other taxa which resembled our *in planta* observation. Surprisingly, under *in vitro* conditions, the carrying capacity for Xp also increased with the increasing growth rate of Xp in mixed cultures, which did not correlate with our *in planta* data, where the carrying capacity for Xp decreased in mixed infections with Pc or Xa or both. These results indicated that host plant environment and the similar nutrient utilization by the three bacterial species plays an important role in reducing the population of the dominant pathogen Xp in mixed infections.

In chapter 2, we observed that along with high humidity inducing bacterial leaf spot disease severity, mixed infection by the co-occurring bacterial species Xa and Pc along with the dominant pathogen Xp also contributed in inducing disease severity. However, the increase in disease severity by mixed infection was not significant. We noticed that when coinfected together, presence of Xa or Pc also contributes to reducing the in planta Xp population. To investigate if Xa and Pc has the same influence on Xp population without the involvement of plant host environment, we performed in-vitro growth curve assay to enumerate Xp population in pure and mixed cultures. Population of Xp was lowest when Xp was by itself in pure culture, whereas in planta Xp population was highest when plants were inoculated by Xp alone.

We observed that Pc promoted the growth and carrying capacity for Xp population in XVM2 media, but this result did not match with our in-planta observation where Pc reduced the carrying capacity and population for Xp. The number of microbial cells that an environment can support is referred as the carrying capacity (McArthur, 2006; Nix et al., 2009). However, presence of Pc increased the Xp growth rate with a reduced doubling time under both in vivo and in planta condition. The Xp growth rate increased possibly as a response of the competition experienced by the presence of Pc. Bacterial growth has been observed to be related to its survival, in a competitive environment (Gurijala & Alexander, 1990). This data correlated with our results that nutritional similarity between Xp & Pc is 0.81 which indicates possible competition for nutrients, but their nutritional similarity was less compared to nutritional similarity between Xp and Xa. The less nutritional similarity between Xp and Pc can be a possible reason of increase in Xp population in presence of Pc because Xp had more available nutrients that were not being utilized by Pc. Results also indicate that competition between Pc and Xp persisted in both in vitro and in planta condition but in absence of the host environment Xp carrying capacity and population did not reduce under

the influence of Pc. It indicates that in presence of Pc, plant host plays a crucial role in causing the reduction of Xp population that cannot be observed in XVM2 media. Probably because the XVM2 can only mimic the plant apoplast environment (Hiery et al., 2013; Sabuquillo & Cubero, 2021), it cannot replicate the heterogeneous leaf surface. Also in vitro conditions do not induce the bacterial species for production of cell wall degrading enzymes to obtain nutrients from plant cells, or PAMP triggered immunity (PTI) in plant hosts. Our in-planta experiment results suggested induction of PTI by Pc which delayed effector triggered susceptibility response or cell death symptom development by Xp in presence of Pc. Therefore, we can assume that lesser nutritional similarity between Xp and Pc (compared to Xp & Xa) was a possible reason for increased *in vitro* Xp population.

The increase in the *in vitro* carrying capacity for Xp and its population caused in presence of Xa was not significant and the Xp growth rate remained almost the same possibly because Xp did not experience competition by Xa as the Xa population was low in the mixed infection but our *in planta* population assay exhibited that presence of Xa caused significant reduction in Xp population and it's carrying capacity. It can be assumed that this difference between *in planta* and *in vitro* data was because of the absence of plant host component in the *in vivo* experiment. Also, if there was any virulence mechanism activated *in planta* to release more nutrients from plant cells by Xa, it was not possible to be detected in XVM2 media (Sabuquillo & Cubero, 2021). Presence of Xa in mixed inoculum barely caused any change in the *in vitro* Xp growth rate. In plants the Xp growth rate increased in presence of Xa under high humid condition possibly as a survival response to the presence of Xa.

We performed bacteriocin production assay, contact dependent inhibition assay to investigate if there were any evidence which would indicate possible competition and antibiosis among the three

bacterial species, but we did not observe results which could confirm antagonistic interactions. Antibiosis can be considered as antagonism mediated by specific or non-specific metabolites of microbial origin, by enzymes or other toxic substances (Fravel, 1988). Even though our *in vitro* data did not necessarily indicate antagonistic relation among Xp, Pc and Xa, the nutritional overlap results among can be considered as definitive evidence for competition among the three species for nutritional resources.

Our genome analysis results showed Xa possess a type III secretion system and can produce AvrBs2, XopZ, XopAL1, XopAW, HrpW, XopAR, XopAD, XpF type III effectors. AvrBs2 has been reported as an essential virulence factor of *Xanthomonas oryzae* pv. *oryzicola* (Li et al., 2015). Type III secretion system (T3SS) allows secretion and delivery of bacterial virulence proteins, called effectors, directly in host cell cytoplasm. Type 2 secretion system secrets most cell wall degrading enzymes like cellulases, poly-galacturonases, xylanases, and proteases (Potnis et al., 2011). COG analysis revealed Xa can produce the principal enzymes required to degrade pectin, lignin, cellulose, hemicellulose present in the plant cell wall. Xa can also produce protease.

All the *in vitro* experiments and analysis indicates that *Pseudomonas* species 93B.260 is a weak colonizer which do not possess primary cell wall degrading enzymes or a complete T3SS system and can possibly produce only few type III effectors. It did not exhibit proteolytic activity and do not have the principal plant cell wall degrading enzyme producing genes, which is also crucial for establishing an infection as plant pathogen. If plants are co-infected with Pc, Xa and the dominant bacterial leaf spot pathogen Xp, then Pc might be benefitted from the presence of the other two species. Pc might colonize better or be able to utilize the nutritional sources which will become available by cell wall degradation caused by Xa and Xp. Pc will also experience less competition

for nutrition utilization. Presence of Pc can also possibly introduce competition in the environment which increases Xp growth rate as a survival response by Xp which resembled our *in planta* observation.

Therefore, from our *in vitro* data we can predict that, in field condition mixed infection by Xa, Pc and Xp might induce a competition for resources among the three bacterial species. To maintain its population density as a survival response to the competition experience by other co-infecting species, Xp will increase its growth rate. These three bacterial species not necessarily inhibit each other by the production of inhibitory substances. Also host colonization might become easier for Xa and Pc in presence of the dominant pathogen Xp.

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4. CHAPTER FOUR

Synopsis

Tomato is one of the most important crops in the world and it is also one of the major vegetables grown in the southeastern United States. *Xanthomonas perforans* is the dominant pathogen that causing bacterial spot disease of tomato. Survey of Alabama tomato fields revealed co-occurrence of *Pseudomonas* species and weak *Xanthomonas* species like *Xanthomonas arboricola* with the dominant pathogen *Xanthomonas perforans*. Association of weak *Xanthomonas* species and *Pseudomonas* species with *Xanthomonas perforans* has also been observed in various other studies. Co-infecting pathogens are considered challenging due to their consequences for individual level infection risk and disease progression. Co-inoculation of 4-week-old tomato plants with mixed inoculum of *Xanthomonas perforans*, *Xanthomonas arboricola* and *Pseudomonas* species under high humidity conditions resulted in higher disease severity but lower *X. perforans* population in the mixed inoculated plants. Nutritional profile analysis of three bacterial species revealed nutritional similarity among the three species with higher similarity between Xa and Xp, which indicated possible competition for resources and explained the reduction in Xp population and carrying capacity in presence of Xa and Pc. As a survival response to the presence of other bacterial species, Xp growth rate increased in mixed inoculums under both *in vitro* and *in planta* conditions. Pc can produce only few type III effectors and lacks Type III secretion system, and it could not suppress PTI (PAMP triggered immunity) induced by itself. The host's PAMP triggered immunity temporarily restricted colonization by Xp in the host and delayed symptom development and effector triggered susceptibility by Xp. Coupled with plant's immune response and competition for resources, population of Xp reduced under mixed infection with Xa and Pc. The growth rate of Xp also increased as a survival response. Therefore, in mixed infection, competition introduced by the co-infecting opportunistic or weak pathogens, can cause the dominant pathogen

(Xp) to increase its growth rate as a survival response. Xa and Pc were able survive the host environment and colonize the apoplast better in presence of the dominant pathogen because Xp makes it easier to colonize by crossing the initial physical defense barrier of plant hosts. Conclusively, our study predicts that farmers can expect to see higher bacterial spot disease severity under high humid condition in fields when there is mixed infection due to the presence of *Pseudomonas* species and *X. arboricola,* but it will not be significant or drastic. Mixed infection will reduce population load and carrying capacity of the dominant pathogen *Xanthomonas perforans* due to passive competition for foliar nutrition, but its growth rate might increase to survive and to maintain the population density. Mixed infection will possibly increase the population of the avirulent pathogens which can be a potential problem as they are opportunistic and colonizes the host better with help of dominant pathogen. Even if crop loss might not significantly induce in case of mixed infection by these co-occurring bacterial species, but it might lead to increasing population of weak opportunistic pathogens, which under high humidity can develop symptoms, like *Xanthomonas arboricola* did.

(Xp – *Xanthomonas perforans* AL 65::Tn7-mre152, Pc – *Pseudomonas* sp. 93B.260::Tn7-mre145, Xa – *Xanthomonas arboricola* CFBP 6826::Tn7-mre160)