Competition between strains of the bacterial plant pathogen *Xylella fastidiosa*

by

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Abstract

Understanding the dynamics of interactions among different strains of the bacterial plant pathogen *Xylella fastidiosa* (*Xf*) is crucial for preventing, detecting, and mitigating potential outbreaks. The potential for recombination and exchange of genetic material among these strains could lead to the emergence of new virulent strains and a wider range of susceptible hosts, making it necessary to investigate these interactions comprehensively. This study aimed to develop strain-specific quantitative PCR (qPCR) primers as a tool to identify and quantify strains of *X. fastidiosa* during mixed bacterial strains cultures and infections. A key consideration to select the competing strains was their classification in subspecies, plant host, and geographic location. The selected strains were *X. fastidiosa* subsp. *fastidiosa* TemeculaL (CA) and WM1-1 (GA) from grapevine, EB92-1 (FL) from elderberry, an avirulent strain, and *X. fastidiosa* subsp. *multiplex* AlmaEM3 (GA) and BB08-1 (FL) from blueberry. Co-culture experiments were performed to evaluate the impact of bacterial competition on growth and survival. Potential mechanisms of competition were assessed using in silico and dual culture methods. Results showed that strain TemeculaL had a significant inhibitory effect on the growth of WM1-1, while strains AlmaEm3, EB92-1, and BB08-1 showed no significant inhibition. Fitness assays revealed that *X. fastidiosa* subsp. *fastidiosa* TemeculaL had an increased growth rate compared to the other strains. The study also evaluated the symptom development in a non-natural plant host, *Nicotiana tabacum* cv. SR1 Petite Havana, and showed high disease progression in plants infected with strains TemeculaL+BB08-1, and low symptom expression in plants infected with EB92-1 and TemeculaL+EB92-1. The latter results support the previous suggestion that the elderberry strain could be a potential biocontrol strain. The study provides new perspectives and innovative approaches for identifying distinctive genetic sequences among different strains of *X. fastidiosa* using novel strain-specific primers.

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List of Abbreviations

PWA	Periwinkle Agar
PD2	Pierce's Disease Media
XF	*Xylella fastidiosa*
CCFF	Co-culture *fastidiosa-fastidiosa*
CCFM	Co-culture *fastidiosa-multiplex*
BLASTn	Basic Local Alignment System Tool Nucleotide
qPCR	Quantitative Polymerase Chain Reaction
bp	basepairs
KEC	K-mer Exclusion by Cross-referencing
NCBI	National Center for Biotechnology Information
CA	California
GA	Georgia
FL	Florida
CmR	Chloramphenicol Resistance Cassette
KmR	Kanamycin Resistance Cassette
OD	Optical Density
CFU/ml	Colony forming Units per milliliter.
CTAB	Cetyltrimethylammonium bromide
EPPO	European and Mediterranean Plant Protection Organization
CAT	Chloramphenicol acetyltransferase
APH(3').	Aminoglycoside 3'-phosphotransferase

Chapter 1

Literature review

Xylella fastidiosa

Xylella fastidiosa (Xf) is an obligate xylem-limited bacterium that exclusively inhabits the xylem vessels of not only susceptible plants but also asymptomatic plant hosts (De Lima et al., 1998). This pathogen can also colonize the mouth parts of sap-feeding insect vectors that acquire and transmit the pathogen. The vector insects that harbor this pathogen are part of the *Cicadellidae* and *Cercopidae* families, commonly known as sharpshooters and spittlebugs, respectively (Hewitt et al., 1946). The bacterium was known for causing Pierce's disease on grapevine, a disease that was first described by Newton Pierce in 1892. *X. fastidiosa* was first named by (Wells et al., 1987), who identified it as belonging to the *Xanthomonadaceae* family. It is classified as a fastidious prokaryote, meaning this bacterium has strict nutritional and growth requirements in order to be cultured *in vitro.* Commonly *X. fastidiosa* is cultured on Periwinkle Wilt (PW), PD2, PD3 medium (Davis et al., 1981) with optimum growth conditions at 26-28^{o} C (Wells et al., 1987). This highly destructive bacterial plant pathogen has been documented to infect a wide range of plant hosts, currently reported to include more than 600 different plant species belonging to 289 genera and 87 families (Delbianco et al., 2021). Its ability to infect a diverse array of plant species has made it a major threat to global agriculture and natural ecosystems.

Life Cycle

The diseases caused by *X. fastidiosa* are typically known to cause leaf-scorch symptoms associated with the extensive colonization of xylem vessels (Chatterjee et al., 2008). Sap-feeding insects, such as sharpshooters and spittlebugs can acquire the bacterium by feeding on infected

plants and later transmit it to healthy plants during the feeding process. The colonization of the xylem tissue results in significant damage to the plant, including wilting, marginal leaf scorching, and eventual death, being this associated with water deficiency and nutritional imbalances (Chatterjee et al., 2008). Xylophagous insects like the glassy winged sharpshooter acquire *X. fastidiosa* through feeding on infected plant xylem sap. *Homalodisca vitripennis* (Glassy winged sharpshooter) have the ability to ingest in excess of 100 times their weight of xylem fluid in a single day (Purcell et al., 1999). Therefore, the high feeding rate of *H. vitripennis* is a significant factor in the spread of *X. fastidiosa* and the impact it has on agricultural and ecological systems. It has been reported that this bacterium can have three retention sites within the vector including the longitudinal groove and the diaphragm/apodemal groove, cibarium chamber, and precibarium (Alves et al., 2008). Later the bacterium is egested with a mixture of saliva into the xylem with the stylet acting as a syringe (Backus et al., 2015). Allowing for the infection cycle to fully take place as shown in (Figure 1).

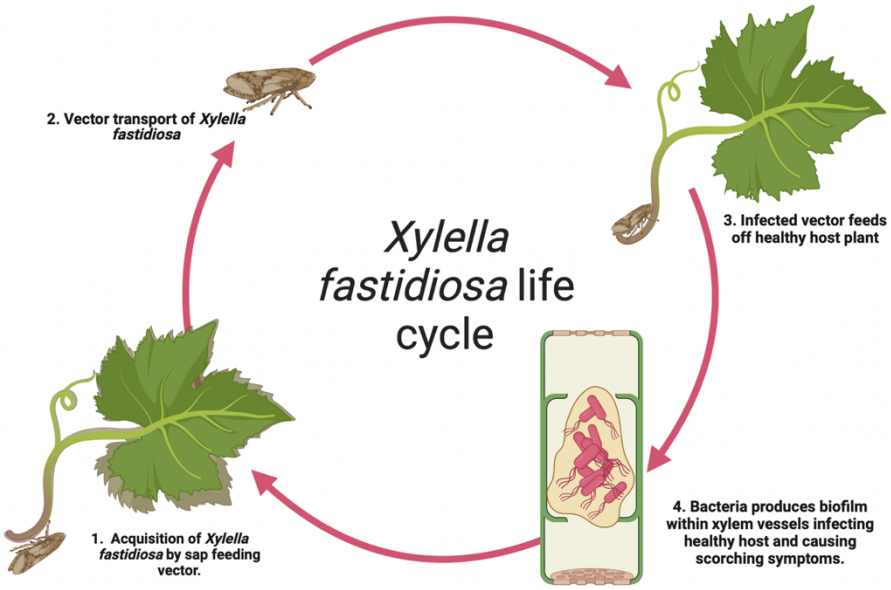

Figure 1. *Xylella fastidiosa* general life cycle from pathogen acquisition to plant infection by xylophagous insects.

Evidence of interactions of *Xylella fastidiosa*

X. fastidiosa was thought to be limited only to the Americas (Almeida and Nunney, 2015), but over the last years it has been found in Europe in Apulia, Italy affecting olives introduced from America (Saponari et al., 2013, Marcelletti and Scortichini, 2016). A distant but related strain was reported from Taiwan, *Xylella taiwanensis* causing pear scorch (Weng et al., 2021). Since 2004 this pathogen was divided into three major subspecies by using DNA-DNA hybridization; *fastidiosa*, *multiplex* and *pauca* (Shaad et al., 2004), with two more subspecies *sandyi* (Vanhove et al., 2019) and *morus* (Nunney et al., 2014, Potnis et al., 2019) thought to have emerged through recombination within subspecies, as shown in (Figure 2). Intraspecies and interspecies interactions of this pathogen signifies a notable concern. Reports indicate that specific strains of *X. fastidiosa* exhibit natural competence (Kung and Almeida 2011), which refers to the ability of bacterial cells to uptake and integrate exogenous fragments of DNA into their own genome. This process facilitates homologous recombination, where the newly acquired genetic material may potentially recombine with the existing genome of the bacterium. Such interactions may result in the emergence of novel strains with altered genetic makeup, posing a considerable threat to the host plant. (Coletta-Filho et al., 2017) reported a bidirectional allelic exchange between subsp. *pauca* and *multiplex* as evidence of homologous recombination in areas where these different genetic clusters occurred sympatrically. Specifically, an allele originating from subsp. *multiplex* was detected in the only strain of subsp. *pauca* isolated from a plum host.

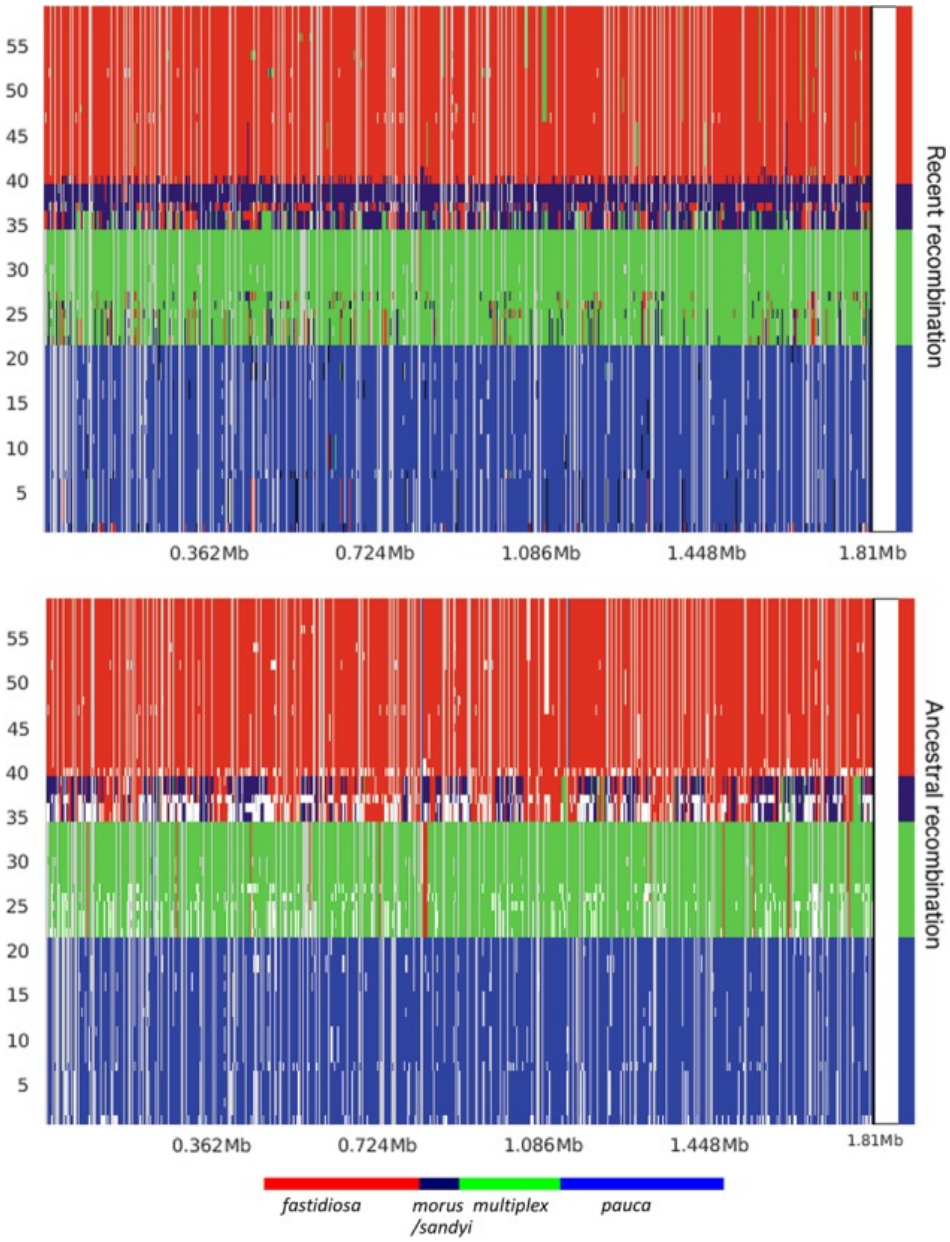

Figure 2. Recombination events in the core genome of *X. fastidiosa* using fastGEAR. Image shows ancestral and recent recombination events, as published by (Potnis et al., 2019).

However, these computational analyses suggest that these strains have entered into a sympatric state which creates conditions conducive for both the occurrence and detection of Interspecies Homologous Recombination (IHR) (Nunney et al., 2014). These computational

analyses suggest that *X. fastidiosa* has undergone several interactions throughout its evolutionary history. These interactions are believed to have played a crucial role in shaping the genomic architecture and functional attributes of this bacterium.

To further support the possibility of these isolates co-existing sympatrically, the first report of co-existence of different genotypes of *X. fastidiosa* was documented in California in almonds (Chen et al., 2005). In that study strains showed different colony morphology, and the study suggested genetic differences among them. Bacteria that co-exist can generally compete for resources or cooperate with each other to form communities, such as biofilms. As a cooperative behavior this applies to bioprocesses in which the efficiency of the process is coupled to the production of shared (public) goods that allow cells to perform tasks in a 'cooperative' manner (Lindemann et al., 2016). Another form of interaction is through antagonism. Microorganisms inhabiting a common environment are known to compete for space and resources (Hibbing et al., 2010). To date, there have been no studies investigating the potential cues for social behaviors among *X. fastidiosa* cells that give us an idea on how these strains interact while coming in contact. Experiments *in vitro* give us important information on an artificial environment under favorable conditions, but conducting *in planta* assays are important to compare to a natural set up. Given the ecological importance of this virulent pathogen and the impact that has left in the agriculture industry, it is a study that must be performed to determine disease outcome.

Commonly used methods for identification and quantification of *Xylella fastidiosa*

Several methods have been available for the identification of *X. fastidiosa*, which include both culture-dependent and culture-independent approaches. Culture-dependent methods involve the axenic growth of a single species, variety, or strain of this bacterium, entirely free of all other

contaminating microorganisms from plant tissue or insect vectors on culture media. Culture-based detection methods on selective media has been proven to be time consuming given the nutritionally demanding nature of the bacterium and inherently it is less sensitive than molecular-based techniques. Culture-independent methods do not rely on the isolation of *X. fastidiosa* in pure culture. Multilocus sequence typing (MLST) has been used for studies of this pathogen (Yuan et al., 2010, Scally et al., 2005, Elbeaino et al., 2014), which is a genetic typing method that uses DNA sequences from multiple genes to identify genotypes of the bacterium. In MLST, several housekeeping genes are sequenced, and compared with sequences of reference strains to determine their genetic relatedness. This method provides a standardized nomenclature for *X. fastidiosa* strains and allows for the comparison of strains from different geographical regions. The MLST approach can provide insight into the population structure and genetic diversity which can be useful in understanding the epidemiology and evolution of the bacterium. But despite being widely used one of the major limitations of MLST is that it targets only a small number of genes, ranging seven to ten, which may not capture the full genetic diversity of a bacterial population.

Another powerful method used for identifying *X. fastidiosa* is the polymerase chain reaction (PCR). PCR-based methods will use designed primers that will amplify a specific user defined region of the genome, allowing for its detection and identification. Previously different approaches used 16S, *gyrB* (Chen et al., 2000, Rodrigues et al., 2003), with others using conserved sequences of the 16S rDNA and 16S-23S internal transcribed spacer were used to design primers (Shaad et al., 2002). A commonly used quantitative PCR (qPCR) uses the genome-based specific primers HL5-HL6 available for the identification of *X. fastidiosa* (Francis et al., 2006). This technique is highly sensitive for small amounts of DNA and specificity can be improved by using fluorescent probes designed for the assay. This molecular based technique allows us to identify

bacteria and quantify them using a specific amplicon. No available reports have been found for strain-specific primers to differentiate *X. fastidiosa* strains from the same or different subspecies using specific identity markers. This will be the first known report to use the pipeline KEC (Beran et al., 2021), that matches one of the main objectives of this research, which is to find that sequence that is only found within the genome of a specific strain. The use of these specific sequences that show no homology towards other strains can serve as a fingerprinting technique to identify and quantify targeted strains of *X. fastidiosa*.

Objectives

General: Understand interactions among *X. fastidiosa* strains.

Specific objectives:

1) Determine outcome of competition between pairs of *X. fastidiosa* strains grown in co-cultures.

- Task 1.1: Perform analysis of co-cultures.
- Task 1.2: Design strain-specific primers for qPCR.

2) Determine the basis of antagonism between *X. fastidiosa* strains

- Task 2.1: Adapt protocols for antagonism studies between fastidious prokaryotes.

3) Study competition of strains *in planta* and their effect on the host

- Task 3.1: Compare symptoms between treatments on tobacco plants
- Task 3.2: Determine bacterial population in plant tissue

Hypothesis

Competition among *X. fastidiosa* strains increases with increasing phylogenetic similarity and niche overlap.

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Chapter 2

Strain-specific qPCR primers for the quantification of *Xylella fastidiosa*

Abstract- Understanding the dynamics of interactions between different strains of bacterial plant pathogens occupying the same niche is a crucial area of research. This is particularly important for *X. fastidiosa* considering their potential to recombine and exchange genetic material, that could result in the emergence of new virulent strains and a wider range of susceptible hosts. This unpredictability poses a significant challenge; therefore, a comprehensive investigation of these interactions is necessary for devising strategies to prevent, detect and mitigate the potential impact of such outbreaks. The objective of this study was to identify distinctive genetic sequences between strains of this bacterial plant pathogen for the development of strain-specific quantitative PCR (qPCR) primers, to investigate the interactions among strains *in vitro* and *in planta*. Strains were selected from different subspecies, host, and geographic location. The strains selected for these experiments were previously isolated from locations within the geographical boundaries of the United States. The cells obtained from the collection and used in this experimental setup were *X. fastidiosa* subsp. *fastidiosa* TemeculaL (CA) and WM1-1 (GA) from grapevine, EB92-1- (FL) from elderberry, an avirulent strain; and *X. fastidiosa* subsp. *multiplex* AlmaEM3 (GA) and BB08-1 (FL) from blueberry. The cells were previously tagged with different antibiotic resistance markers to assess culturable populations under competition. Additionally, using the bioinformatic pipeline KEC (K-mer Exclusion by Cross-referencing), non-shared sequences between the experimental strains were acquired and sequences were confirmed present/absent using BLASTn to design qPCR strain-specific primers. As a result, 3 out of the 5 experimental strains showed presence of the extracted non-shared sequences. Primers were designed around the internal sequence of a hypothetical protein in AlmaEm3, and intergenic sequence in EB92 and a putative

gene coding for endonuclease subunit S specifically found within the genome of BB08-1. These promising genetic markers identify these strains by qPCR with primers within the acceptable efficiency range of 90-110%. Overall, this study offers new perspectives and innovative approaches for the identification of these strains with novel strain-specific primers.

Introduction

Xylella fastidiosa (Xf) is a vector-transmitted bacterial plant pathogen that colonizes the xylem vessels, infecting more than 600 plants (EFSA, 2021). Its virulence mechanism involves the formation of biofilm that clogs the xylem vessels, causing symptoms of chlorosis and scorching by impeding the flow of water from root to shoot. It is a member of the *Xanthomonadaceae* family (Wells et al.,1987) and is vectored and transmitted by xylem-feeding insects, such as spittlebugs and sharpshooters. *X. fastidiosa* is known for its high genetic diversity and ability to rapidly adapt to new hosts and environments. It has been identified as the causative agent of several plant diseases, including Pierce's disease of grapevine (Pierce, 1896), citrus variegated chlorosis (Schaad et al., 2004), and in 2013 identified in olives causing olive quick decline syndrome (Saponari et al., 2013). A typical symptomatology for this bacterium is plant stunting, leaf scorch, fruit shriveling, leaf cupping and ultimate death of infected plants, which leads to significant economic losses for the agriculture industry. Recently it has been estimated that in Italy alone if the disease spreads over the course of 50 years it is expected that damages by this pathogen could range from 4.1 to 10.3 billion Euros (Schneider et al., 2021). *X. fastidiosa* is a concern for the environment, as it can have a significant impact on natural ecosystems and affect plant biodiversity. There has been no described cure for *X. fastidiosa* and management strategies resort to the prevention of the spread of the bacterium through strict quarantine measures of plant material, use of insecticides to

control insect vector population (Wilson et al., 2020), and removal and destruction of infected plants. Given the severity of infections in agricultural important crops, research efforts are focused on developing new tools for rapid detection for easy tracking of the spread of the bacterium, as well as identifying potential targets for control measures. This chapter is focused on developing strain-specific detection and quantification methods with the use of bioinformatic tools and molecular techniques, focusing on strains found mainly in the United States.

Materials and methods

Bacterial strains, culture conditions and DNA extraction

Strains used in this experiment were previously isolated in the United States and later transformed with antibiotic resistance markers (Kandel et al., 2017). For the experiments conducted in this chapter we selected TemeculaL-NS1-CmR, WM1-1-pAX1-KmR, Alma-EM3-pAX1-KmR, EB92-1-pAX1-KmR and BB08-1-pAX1-KmR who were previously tagged with a suicide plasmid (Matsumoto et al., 2009) designed to insert itself into the Neutral Site (NS1) of *X. fastidiosa.* The cells were cultured directly from frozen 25% glycerol stocks on Periwinkle Agar (PWA) containing 10µg/ml of Chloramphenicol or 30µg/ml of Kanamycin (Kandel et al., 2017) (depending on the mutant antibiotic resistance), incubated at 28^{o}C for 7 days and later re-streak for experiments. Bacterial cells were suspended in PD2 broth and adjusted to an OD_{600} of 0.5 for DNA extraction. The DNA was extracted by CTAB method as previously described (EPPO Bulletin, 2019) with adaptations of the plant extraction protocol for an *in vitro* experimental set up.

Genomic dataset

A collection of 127 *X. fastidiosa* (taxid:2371) genomes were obtained from the National Center for Biotechnology Information (NCBI) and subsequently compiled into a directory for thorough analysis. To ensure a diverse representation of the species, a selection of five experimental strains were chosen from specific subspecies, hosts, and geographic locations found in the United States.

Non-shared sequence extraction

A K-mer Exclusion by Cross-referencing (KEC) pipeline, as developed by (Beran et al., 2021), was utilized to recover non-shared sequences from a large genomic dataset. The specific objective was to identify unique sequences that could be potentially used for primer design in diagnostic and quantitative qPCR assays. The target genomes used in this experiment were separated into a distinct (Target) directory and compared to the remaining genomic dataset (Non-Target) using the KEC pipeline's exclude mode to retrieve the desired target sequences. Typically, we aimed to determine the smallest k-mer size that would yield results. This was accomplished by commencing with a number approximately equal to 12 as recommended by the developer and then either increasing or decreasing it until we found the smallest number that produced more than 0 sequences as suggested by (Beran et al., 2021). For this analysis, k-mer sizes that yielded results were k=13 used for TemeculaL and WM1-1, while k=20 was used for Alma-EM3, and k=12 for EB92-1 and BB08-1.

Sequence alignment

The retrieved sequences were subjected to further analysis. The first step involved converting the sequences into the FASTA format using an online tool (https://www.hiv.lanl.gov/content/sequence/FORMAT_CONVERSION/form.html), followed by uploading them into a specific directory for subsequent analysis. As a second step for the analysis, a nucleotide BLAST database was created for each group of sequences uploaded. Third step, the retrieved sequences for all five experimental strains were aligned against the complete set of 127 *X. fastidiosa* genomes to identify any sequences that were exclusive to the experimental target genomes. This allowed for the identification of unique candidate sequences that could be potentially useful for diagnostic qPCR assays. Subsequently, the resulting sequences were evaluated for their length (>70bp) to ensure they were suitable for primer design.

Unique primer design and cross alignment for primer specificity

Candidate sequences were selected based on alignment specificity to the experimental strain. Primer pairs were designed on the Integrated DNA technologies website using the online tool "Primer Quest" (https://www.idtdna.com/pages/tools/primerquest) and selecting the option for Intercalating dye. Number of primer pairs was completely dependent on the length of the sequence. Primers were designed regarding sequence length with a minimum size amplicon of 75 bp to a maximum of 200 bp. Designed primers were subjected to quality testing for the analysis for self-annealing, hairpins, and GC content. Primer pairs that passed with quality test were later subjected to alignment using Primer Blast to verify amplicon size, potential secondary amplicons within the genome that could range in sequence length, theoretical Tm and was also cross aligned to other experimental genomes to confirm specificity to the intended target. After specificity was confirmed the primer pairs were ordered and used for experiments. In the case of strains for which

unique sequences were not identified, the antibiotic resistance cassettes inserted as markers were used as targets for identification. Those sequences were searched with KEC using the genome sequence of the mutant strains and the sequence was confirmed to be the resistance gene by using ORF finder.

Primer specificity *in vitro* cross reaction assay

To carry out the PCR assays, primers were prepared as followed: lyophilized primers were suspended and dissolved with sterile nuclease free water to a concentration of 100µM to create a stock solution under aseptic conditions. For the working stock, a separate aliquot was taken from the stock solution and then diluted again with sterile nuclease-free water to a concentration of 10µM in a separate sterile 1.5ml microtube. Primers and all equipment used regarding the PCR setup were treated with strict sterility protocols to prevent contamination by foreign DNA and ensure accuracy of the results. Sterile filtered tips were utilized during the procedures to minimize the risk of any potential contamination. The PCR parameters used for this assay were carried out as follows: initial denaturing at 95ºC for 3 minutes, denature 95ºC for 30 seconds and then the annealing/extension was combined into a single step with an optimum temperature of 65ºC for 45 seconds. Most of the primers their annealing temperature were within 3°C of the extension temperature, making this assay a two-step PCR in which both steps are combined, and the total time of the entire assay is shortened. A cross-reactivity assay of the PCR reactions was performed on both Bio-Rad systems, S1000 Thermal Cycler with Taq 5X Master Mix from New England Biolabs and the CFX96 Real-Time System using Sso Advanced Universal SYBR green supermix. Amplicons were run on a 1.2% of agarose gel at 110v for 45-60 minutes and stained with Gel Red Nucleic Acid by Biotium for visualization.

qPCR primer efficiency

To determine the qPCR primer efficiency, previously extracted gDNA of the experimental strains was aliquoted and a ten-fold dilution series was prepared. Subsequently, reaction mixtures were prepared for each dilution series and amplified using the designed primers. The Ct values for each dilution were recorded and plotted against the Log10 Genome Equivalents. By using the standard curve equations, the efficiency of the reaction mixture was calculated using the Efficiency formula $E= (10^{-1/slope}-1) \times 100$. Amplification efficiency, as determined from the slope, should range between 90% and 110% (Taylor et al., 2019).

Results

Unique sequence extraction with Kmer Exclusion by Cross-referencing (KEC)

Once the dataset of 127 genomes of *X. fastidiosa* was assembled, the experimental strains TemeculaL, WM1-1, AlmaEM3, EB92-1 and BB08-1 were placed in their specific directories and later compared to a different directory (non-Target) that contained the remaining genomes except the target genome. Each experimental strain had their own target directory and nontarget that excluded them from the list. This resulted in the pipeline finding unique sequences and created an output folder named [*Strain name* unique]. All strains yielded sequences with some strains (AlmaEm3 *k=20*, EB92-1 *k=12,* BB08-1 *k=12*) the k-mer sizes used were different due to the pipeline not finding sequences using the suggested default *k*=12 (Table 1). We wanted to find the lowest k-mer size that produces any results. We achieved this by starting at a number around 12 and increase or decrease the number until the lowest number producing more than 0 sequences was found (Beran et al., 2021).

Table 1. Total number of extracted sequences from experimental *X. fastidiosa* genomes using KEC.

Strains	Sequences extracted
TemeculaL	11
WM1-1	1
Alma-EM3	11
EB92-1	2
BB08-1	8

Sequence alignment

All sequences obtained from KEC were aligned to all the *X. fastidiosa* genomes used in this study using BLASTn on a command line to determine presence/absence. Sequences from TemeculaL, WM1-1, BB08-1 only showed hits to the intended target genome apart from AlmaEM3 and EB92-1 having their sequences being homologous with other off target hits of strains that were not used in this project, making them candidates for novel primer design for our experimental setting as shown in (Table 2).

Table 2. Sequence hits to the intended target genomes

Strains	Sequence ID	Genome match
TemeculaL	#2	**TemeculaL***
WM1-1	#1	**WM1-1***
Alma-EM3	#9	**Alma-EM3***, BB01-1, BB164
EB92-1	#1	**EB92-1***, CFBP (7969, 8078,8082)
BB08-1	#5	**BB08-1***

Note: (*) and bold letters are the intended target strains.

As a limitation to this pipeline, the sequences are extracted based on uniqueness to the target strain and not by possible function. *X. fastidiosa* TemeculaL matched a 456bp contig with accession number (NZ_PUJJ01000130.1). with no annotation available. The sequence showed a 100% query coverage and identity to the intended target strain and possessing only one copy within

the genome. WM1-1 matched a 390bp contig with accession number (NZ_PUJK01000023.1). The sequence showed 100% query coverage and identity and is annotated as acyl coA fatty acid. AlmaEM3 has a partial sequence from a hypothetical protein with a locus tag C5H23_11305 that shows homology with three subspecies *multiplex* strains. EB92-1 has an intergenic region between XFEB_RS12625 (hypothetical protein) and XFEB_RS03460 (hypothetical protein). BB08-1 has an internal sequence for a putative gene annotated as restriction endonuclease subunit S with a locus tag C5H22_01360. After sequence alignment, the main objective was to select candidates following these criteria for qPCR primer design: (1) Ideal sequence length >70bp; this allows for multiple primer pairs to be designed on different positions and be tested for amplification of the intended target, (2) Sequences should only show one hit within the genome and not multiple copies. One copy of the target sequence will result in a more exact number that can be translated into bacterial numbers. Multiple copies of these sequences may artificially increase bacterial counts by using qPCR. Normally bacterial counting assays dependent on qPCR are centered in 16sRNA but it has been shown that since 16sRNA has variable copies within bacterial species this number cannot be translated directly into bacterial counts (Klappenbach et al., 2000, Smith and Osborn, 2009). This same parameter was used for this analysis. The sequences that showed parameters as mentioned previously were the ones selected for primer design as shown in (Table 3).

Table 3. Candidate sequences extracted by the KEC pipeline.

Strains	Candidate sequence (5'-3')	Putative gene
TemeculaL	TGTGTGAGTAGTCGTACTGAGTGCGGCCGCGGGACAAGGTGTCCCGTGGCT AGGCTAATTTGCGTTCCACCTCGGTGCCGAATCATTCAAATTAATGCGCAG TTTTTCAATACGAAATCTCTTCTAACAAACAAACCCGGCATTGCCAATTCCA AATGCGCACAGTGGTTGGTGACAAGTGGTGCGCATTTGTTGGGAATTCTTT TCATATTTTTAAACCTATTAATTAAATTATTGTGGCCCGAGTGAATGTGAAT TAGACTAGTTGCGGACACTGTAGTGCCCACGGCTGTGCTCTTCTGTTCTAAT TAAGAGTCCAATAATTTAATCAGTTTAATTTAGTTGATTAAATCAGTCATCT GAGCAAGGGGTGGCTACCTGAGAGCCGATTACAAACATAGAATCTAGCAT TGTTTGGGTCAACACCGTAGACCGTCTCTGTGGTTCGACTCCGGT	Sequence is a short contig consisting of 456bp. Accession number (NZ_PUJJ01000130.1). No annotation within this contig found.
WM1-1	CCGTGACAGCCCCTGGCTGCGCCAAATGCACGACGGCGCCCGGCACCTCGC CATATTTCTCATCCGCCAGACCGAACACCGCCGCTTCGGCAACCTCGGGAT GCTGGTAAAGCGCATCCTCGACTTCCTGGCAGCTGATGTTCTCACCGCCAC GAATGATGATGTCCTTTTTGCGATCCACGATGAAGAGGTAGCCGTCCTCGT CGAGGTAGCCGATGTCGCCGGTGCGAAAATATCCGTCGGCGGTGTAGCAG GCCTTGGTCGCCTCGGCGTTGCCCCAATATTCCTGGAAGCAGGCGGCCGAG CGGATCGAGATTTCGCCCCGTTCACCCTGTGGCACCGGCGCTCCGGCATCG TCGAGGATGGCAAGATCCACCAGCGGCATGGAGG	Sequence is a short sequence consisting of 390bp. Accession number (NZ_PUJK01000023.1). Sequence is annotated as acyl coA fatty acid.
AlmaEM3	ACGGCGCGCCCAAACTTGAGCATTACGACTACGACCTCAACGCCTTTCTAG ATGCACGCATTAATCATCTGCGGCAACAATGGCA	Partial sequence obtained from locus tag C5H23_11305. Sequence is annotated as a hypothetical protein.
EB92-1	AGTACAGGATACCCCAGCCGAAGAAGTACAGCCCACCTCAGCCGTATCAG CACCACACGAGTTTACAGCCGATACACCAAAGGCAGGTGAATCATGAC	Intergenic region between XFEB_RS12625 (hypothetical protein) and XFEB_RS03460 (hypothetical protein).
BB08-1	CAAGGCGTCGGATTACCCTTCCTTCAAGGATGTGCAGAGTTTGGAGCCACA GTTCCAAAGCCTGTTCAGTTTTGTCCTACTGCAAGCAAGAAGGCTCCCATC AACTCGATCTTGATGTCGGTTCGAGCTCCAGTAGGCAAGATGAATATGGCA GATCAGATGTACGGGATTGGGCGGGGGCTGTGCTCCGTTGCACCAAAGAAT GCAGTATGGCTAACGGATTTTGCACGTTTTGCGCTTGAGAGCGGTGCCGGG CAGCTTAAGTATTTAACGACTGGATCGACGT	Internal sequence of restriction endonuclease subunit S. Locus tag C5H22_01360.

Primer Design and specificity

The sequences chosen for primer design were analyzed using PrimerQuest from Integrated DNA Technologies. Several candidate primers were designed and later subjected to quality testing for possible self-annealing and hairpin turns. Primers that passed the quality testing were selected for *in silico* analysis using PrimerBlast to obtain high-quality primers that are specific to the target sequence and allowed for the identification of potential off-target hits. Using this tool provided the expected amplicon size, as well as the likelihood of variable amplicons that may result from primer binding to other regions within the genome. Primer pairs that showed only one hit to the intended target were selected for future experimental assays. Cross-reactions *in silico* was also performed showing specific binding to the intended genome. After testing, 5 primer pairs were able to be determined *in silico* to be specific with amplicons raging from as low as 76bp- 110bp as shown in (Table 4).

Table 4. Candidate primers for experimental *X. fastidiosa* strains.

Strains	Primers	Amplicon
TemeculaL	T2AF: GGCTGTGCTCTTCTGTTCTAAT T2AR: GTTTGTAATCGGCTCTCAGGTAG	110bp
WM1-1	WM1F: GGCACCTCGCCATATTTCT WM1R: GAAGTCGAGGATGCGCTTTA	86bp
AlmaEM3	AF9: CGCCCAAACTTGAGCATTAC AR9: CATTGTTGCCGCAGATGATT	76bp
EB92-1	EB92F: CCCAGCCGAAGAAGTACAG EB92R: TCACCTGCCTTTGGTGTATC	78bp
BB08-1	BB08F: CGTCGGATTACCCTTCCTTC BB08R: GAGTTGATGGGAGCCTTCTT	102bp

Using gDNA from the experimental strains, the cross- reactivity assays were carried on 1.2% agarose gel electrophoresis and stained with Gel Red to a concentration of 1X as recommended by the manufacturer. Post electrophoresis the gel was visualized using a UV trans

light. Upon completion of the electrophoresis, the amplicons produced by three out of the five primer pairs were found to exhibit the desired specificity towards their target sequences, with their amplicon sizes matching the expected range as shown also in the *in silico* testing. However, primer pairs from TemeculaL and WM1-1 did not exhibit any amplification, neither towards the intended target strain nor any of the other strains tested. Conversely, strains AlmaEm3, EB92-1, and BB08-1 exhibited the desired specificity towards their respective targets. Strains TemeculaL and BB08-1 were later subjected to a gradient PCR (data not shown) with BB08-1 primer pairs and successfully discarded the off-target amplification shown in the well 26 of TemeculaL (Figure 1).

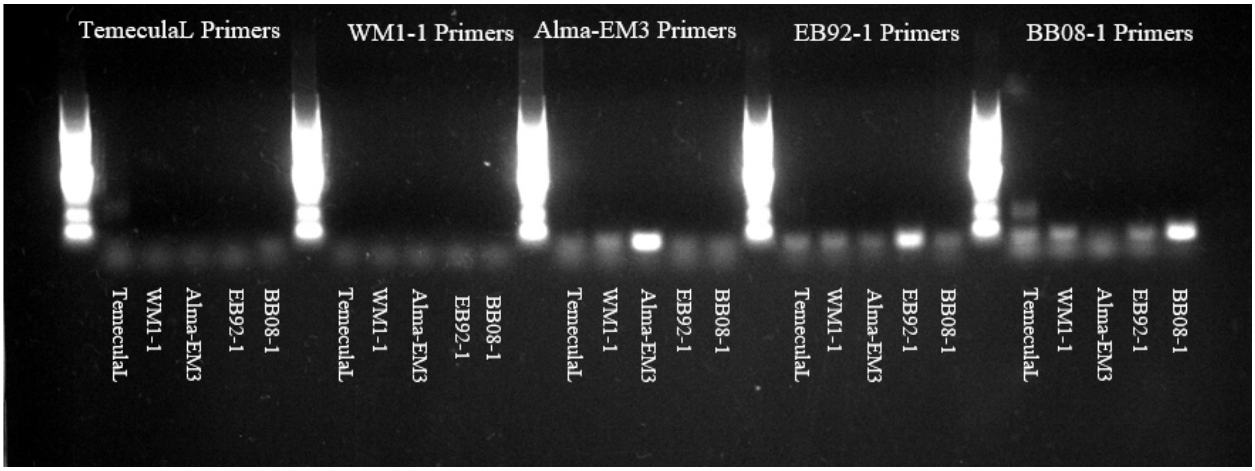

Figure 1. Primer specificity tested by PCR. Cross-reactivity assay was performed by PCR and amplified fragments were analyzed by electrophoresis on 1.2% agarose gel. Primers were designed for specific recognition of experimental strains used in this study.

Following the generation of multiple reaction mixtures increasing $MgCl_2$ concentration in 0.5mM increments the amplification was not successful. Sequences were anticipated to be present with the results of BLASTn alignment but were not detected by the PCR assays concluding the sequence was not present within the genome. In order to obtain sequences and primer pairs for

TemeculaL-NS1-CmR and WM1-1-pAX1-KmR, KEC was used to obtain the specific sequence for these antibiotic resistance cassettes. The sequences were later analyzed using ORF finder to determine the annotation of each sequence, concluding that the sequences coded for Chloramphenicol acetyltransferase (CAT) for Cm^R and Aminoglycoside 3'-phosphotransferase APH (3') for Km^R antibiotic resistance. The sequences were used for primer design and a cross-reactivity assay following parameters mentioned above. Primer pairs shown in (Table 5). Primer pairs showed specificity towards the antibiotic resistant mutants in the cross-reactivity assay with Cm^R yielding the expected size amplicon of 97bp and the Km^R right around 87bp as shown in (Figure 2).

Table 5. Antibiotic resistance primers for strains with absent unique sequences.

Strains	Primers	Amplicon
TemeculaL	CmRF2: AGAAACTGCCGGAAATCGT	97bp
	CmRR2: GGGATAGTGTTCACCCTTGTT	
WM1-1	KmRF4: ATGCTTGATGGTCGGAAGAG	83bp
	KmRR4: GTAGCGTTGCCAATGATGTTAC	

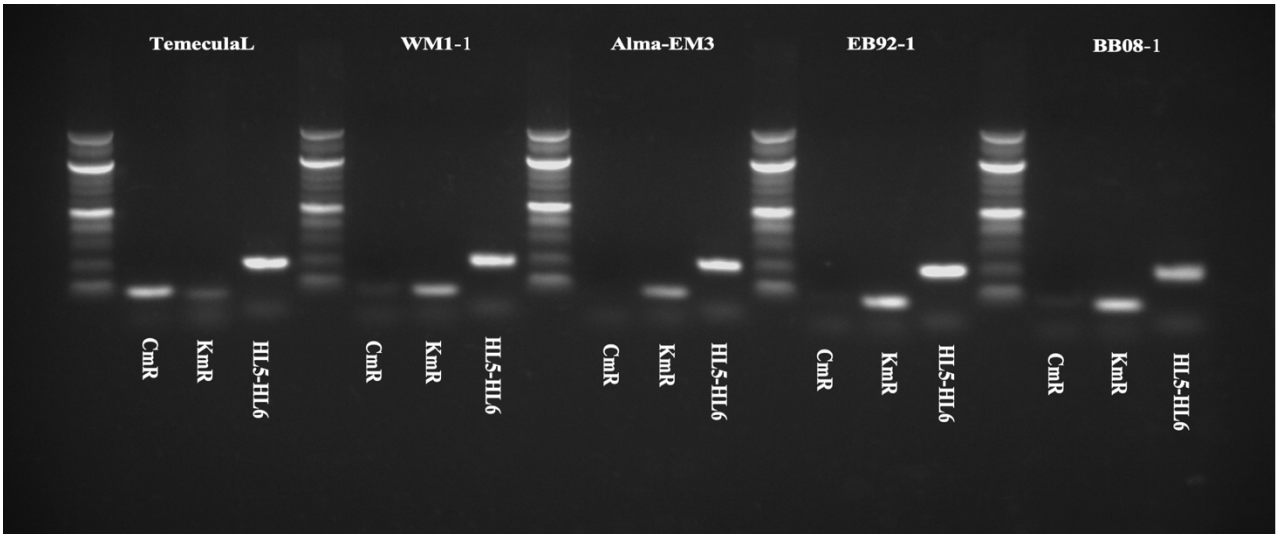

Figure 2. Cross-reactivity assays performed to determine specificity of the secondary primers on all experimental strains to differentiate strains by antibiotic resistance with HL5-HL6 primers

(Francis et al., 2006) as a positive control used to indicate presence of DNA in the sample and a known amplicon.

By validating the primer specificity using conventional PCR, the same assay was carried out using qPCR analysis resulting in specificity of the primers with their desired targets with low Ct values. As expected, the Ct was low for the intended targets meaning specificity was achieved with the primers for the target they were designed as shown in (Table 6). This value is inversely proportional to the amount of target nucleic acid in the sample suggesting that low Ct values indicate a large amount of target DNA present in the sample.

Table 6. qPCR primers cross reactivity reactions

	Average Ct Values						
Strains	**TemL Primers**	**WM1-1 Primers**	**AE3 Primers**	**EB92-1 Primers**	**BB08-1 Primers**	**CmR**	**KmR**
TemeculaL	N/A	N/A	33.019	N/A	34.29	**14.86***	30
WM1-1	N/A	N/A	37.35	N/A	38.3	36.35	**16.40 ***
Alma-EM3	N/A	N/A	**18.81***	N/A	N/A	30.4	**14.85***
EB92-1	N/A	N/A	N/A	**16.60***	N/A	N/A	**16.66***
BB08-1	32.62	N/A	N/A	35.16	**14.094***	N/A	**14.00**

Note (*): Expected Cycle threshold value to determine primer specificity.

Primer Efficiency

The efficiency of the designed primers pairs was evaluated using real-time qPCR coupled with SYBR green as a fluorescence marker. This involved amplification of the tenfold diluted target DNA of a known starting concentration of 100ng/µl. This DNA concentration worked best for qPCR as in comparison to lower concentrations previously used (2ng/µl) in which primer pairs were showing higher Ct values in concentrated samples. The amplification efficiency (E) percentage of efficiency (E%) was calculated using the standard curve method with the data collected from the qPCR runs (Figure 3), and the results indicated that all the primer pairs exhibited high amplification efficiencies falling between the acceptable range of 90-110% as shown in (Table 7 and Figure 3). These findings suggest that the designed primers are highly efficient and specific and can be effectively used for the detection and quantification of the target strains.

Table 7. qPCR primer efficiency and standard curve parameters for novel primers

***X. fastidiosa* Strains**	**Sequence target**	**Efficiency**	**R^2**	**Slope**
TemeculaL	CmR	106%	.9978	-3.1825
WM1-1	KmR	101%	.979	-3.2898
Alma-EM3	#9	108%	.9694	-3.1351
EB92-1	#1	104%	.9407	-3.2368
BB08-1	#5	103%	.9887	-3.2507

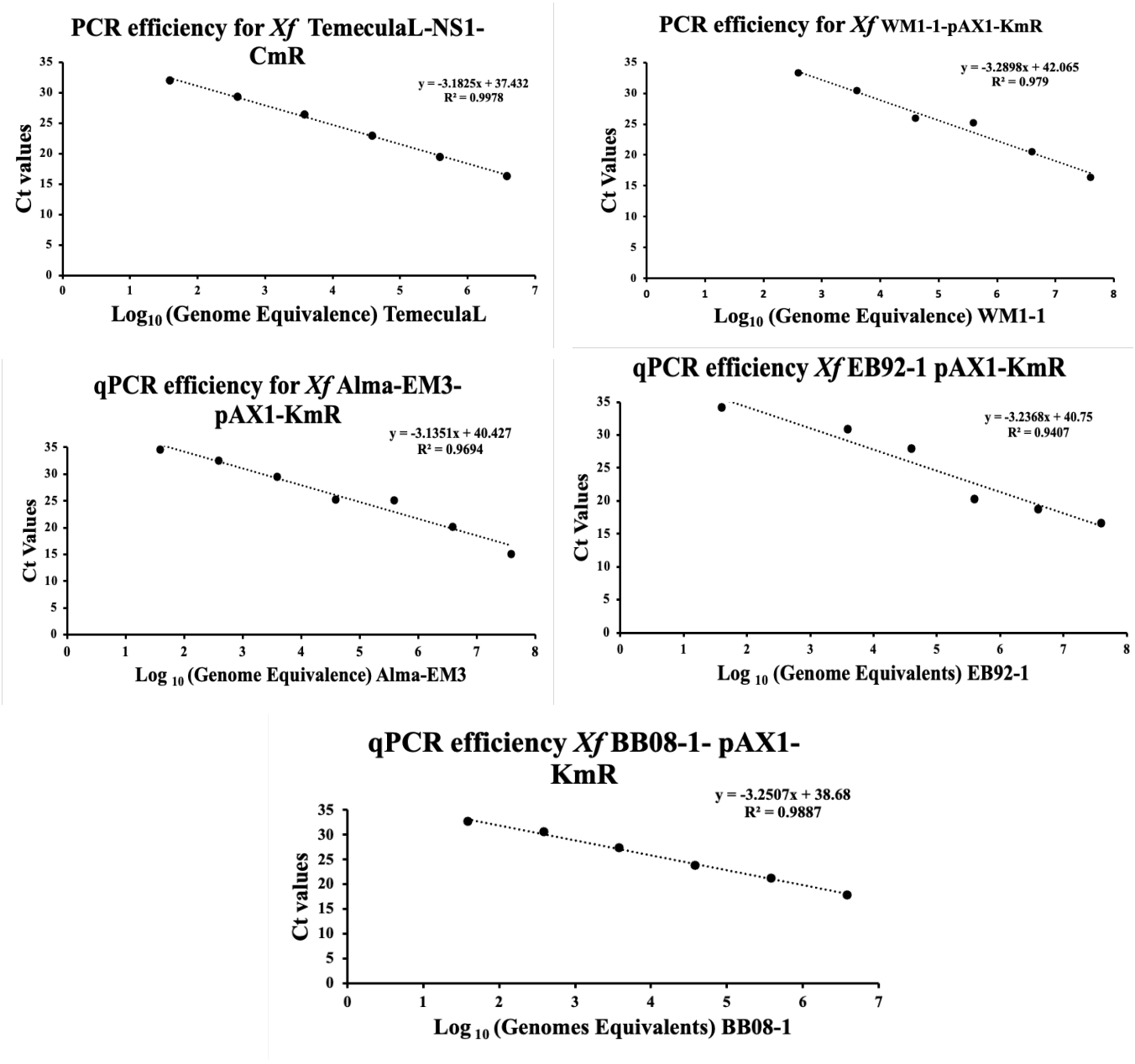

Figure 3. Standard curves for novel experimental *X. fastidiosa* qPCR primers. The curves show the relationship between the logarithm of the initial template DNA of a known concentration and the corresponding cycle threshold (Ct) values obtained for each dilution of the template DNA.

DISCUSSION

The detection of *X. fastidiosa* has been an essential task due to its devastating effects to crops of economic importance. The early detection of this pathogen has been proven as an essential tool in the management of *X. fastidiosa* outbreaks. One of the most employed methods for general detection of *X. fastidiosa* is a validated set of genome-based primers (Francis et al., 2006). These primers are designed to target a conserved 221bp region that is universally present across all strains of *X. fastidiosa*, thereby enabling a reliable and accurate detection of the bacterium. Other approaches by previous researchers include using RAPD-PCR targeting Oleander Scorch strains (Huang, 2009). These amplicons were generated by Random Amplified Polymorphic DNA (RAPD), and the markers were generated through PCR amplification of random segments of genomic DNA using single primers with arbitrary nucleotide sequences. A disadvantage to this technique is the low reproducibility mainly because PCR parameters must be consistent between experiments. A low DNA concentration affects the reaction by decreasing the amplification efficiency thus creating false negative results and complex patterns of RAPD also prevent mixture interpretation and provide challenges in consistent scoring of electrophoretic images even in single-source samples (Butler, 2012). Other approaches focused on detecting *X. fastidiosa* have been limited at the subspecies level. Multiplexing qPCR assays for the simultaneous detection of *X. fastidiosa* (Dupas et al., 2019). (Hernandez-Martinez et al., 2006) developed primers to detect by conventional PCR subspecies *multiplex*, *fastidiosa* and *sandyi*, making them not suitable for qPCR analysis due to the large amplicons produced and not specificic between experimental strains. (Burbank and Ortega, 2018) designed primers to detect subspecies *fastidiosa* and *multiplex*. Since most of these assays target sequences shared by multiple strains, they are not useful to

differentiate between closely related strains. This is where strain-specific primers play a crucial role.

In this investigation, the combination of a new bioinformatic tool KEC developed by (Beran et al., 2021) with BLAST allowed for targeting a unique sequence that is specific to a particular strain. As done by several protocols cited above, the use of BLASTn for *in silico* analysis and the experimental validation using conventional PCR and qPCR is needed. The presence of these sequences was successfully confirmed in 3 out of the 5 strains; AlmaEM3 (Oliver et al., 2014), EB92-1 (Zhang et al., 2011, Zhang et al., 2015), and BB08-1 (Donald Hopkins University of Florida, Oliver et al., 2014) with primers that only work on their intended targets with their pre-determined amplicon sizes. For the strains TemeculaL (Potnis et al., 2019) and WM1-1(Parker et al., 2012) the lack of detection of the expected PCR products, despite the identification of the target sequences using BLAST and *in silico* validated using Primer Blast with the target genome as a template sequence also called in the software as "Custom Database". Computational analysis indicated that the sequences were present but in the experimental set up no amplification was evident. Troubleshooting measures including temperature gradients and $MgCl_2$ optimization were performed to evaluate for non-specific binding in the assay. It was concluded that experimentally the sequences are not present within the genomes of both *X. fastidiosa* TemeculaL and *X. fastidiosa* WM1-1 possibly due to assembly errors that can lead to the absence of target sequences on a physical experimental level.

This drawback meant that the strains didn't yield any specific amplicons. Two solutions are proposed. First, potential targets for TemeculaL may be searched using the reference genome

of Temecula1(AE009442.1) with sequences that share homology between two strains. Second, which was carried out in this project, is the use of the already known antibiotic markers present in the genome from the suicide plasmid developed by (Matsumoto et al., 2009). These were confirmed to work in a conventional PCR and qPCR setup.

The results of this study have several significant implications on the development of novel primers to identify this bacterium at the strain level. This workflow provides an opportunity of more targets strains to be analyzed and tested for strain dynamics and diagnostic purposes. To date, this is the first report of the use of this pipeline to detect unique targets for *X. fastidiosa* at the strain level. This reliable and accessible technique has proven to be a powerful tool for the early detection and quantification of bacterial infections as well as to minimize the negative ecological impacts caused by this pathogen.

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Chapter 3

Competition among strains of the bacterial plant pathogen *Xylella fastidiosa*

Abstract- Bacterial competition is an important factor in microbial ecology and determines the evolution of successful microbial communities. *X. fastidiosa* is a vector-transmitted plant pathogenic bacterium, causes significant damage to economically important crops worldwide. In this study, the interaction of 5 strains of *X. fastidiosa* (TemeculaL, WM1-1, AlmaEm3, EB92-1, BB08-1) was assessed in synthetic culture media and *in planta.* Using co-culture experiments, the interactions were evaluated to determine the outcome of the strains in a communal environment. Results showed that strain TemeculaL had a significant inhibitory effect on the growth of WM1-1, while strains AlmaEm3, EB92-1 and BB08-1 were not significantly inhibited by TemeculaL as both cells grew together. Inhibition was seen in phylogenetically close strains that shared the same host. Potential mechanisms of competition, such as the prediction of biosynthetic gene clusters regarding antimicrobials, was assessed by a computational approach with AntiSMASH 7.0 and BAGEL4 and by dual culture of both bacteria. AntiSMASH 7.0 did not predict any biosynthetic gene cluster, whereas BAGEL4 identified a cluster that encodes for Colicin V. Inhibitory halos were not detected under the conditions tested. To assess if the inhibition observed was caused by nutrient competition, the growth rate of each bacterium was assessed for a period of 7 days. *X. fastidiosa* TemeculaL showed an increased growth rate when compared to the other strains. Competition *in planta* experiments were performed using the non-natural plant host *Nicotiana tabacum* cv. SR1 Petite Havana. The development of "apical leaf scorching" symptoms was evaluated for a period of 9 weeks on plants that were infected with one or two strains. Bacterial quantification was performed as expected due to noisy data in the qPCR experimental setup.

Disease severity curve and AUDPC analysis showed a high disease progression with plants infected with strains TemeculaL+BB08-1 (29.14%) and a low symptom expression in the plants infected with EB92-1 (5.14%) and TemeculaL+EB92-1(3.72%) suggesting that the elderberry strain reduces symptoms, making it a possible biocontrol strain as previous studies suggest. Further studies are needed to elucidate the mechanisms underlying bacterial competition *in vitro* and in the plant host environment.

Introduction

Xylella fastidiosa is a gram-negative, xylem-limited bacterium that is vectored by sap feeding insects and responsible for causing a range of diseases in plants, including Pierce's disease in grapevines, bacterial leaf scorch in blueberry, and leaf scorch in a variety of other plant species. *Xylella fastidiosa* has a persistent and propagative (but non-circulative) relationship in adult sharpshooter insects, which is the only described vector–microbe relationship of this sort (Rapicavoli et al., 2018). This pathogen cannot live outside either the vector or the plant thus it colonizes the mouthparts of the vector and the host xylem (Rapicavoli et al., 2018). It possesses a wide plant host range expanding to more than 600 plant hosts (EFSA, 2021).

Some *X. fastidiosa* strains are naturally competent, meaning that they can acquire foreign DNA with ease and incorporate it into their genetic repertoire allowing for new virulence traits and an unprecedented exchange of genetic material. This makes possible the appearance of new recombinant strains, and the possibility of co-existence of different subspecies within the same geographical location can influence recombination within the subspecies. Reports of genetic introgression have been documented in Brazil in which homologous recombination played a role

in the shift of *X. fastidiosa* subsps. *pauca* from its unknown host to citrus and coffee (Nunney et al., 2012). Further studies (Coletta-Filho et al., 2017) supported that recombination is important for the evolution of this pathogen for new host pathogen associations. The transport of this pathogen is dependent on either infected plants or vectors that feed on them making the encounter of different geographically distinct strains highly probable. This bacterium is classified mainly in three subspecies; *fastidiosa, multiplex* and *pauca* (Schaad et al., 2004). And recombination has been reported within the subspecies showing different recombination events in specific positions of the core genome (Potnis et al., 2019). For some of these recombination events to happen the bacteria somehow have to come in contact one with the other. The first report of two different genotypes of *X. fastidiosa* within the same almond orchard was reported in 2005 in California (Chen et al., 2005), Netherlands in Coffee plants imported from Central America (Bergsma-Vlami et al., 2017) and France in *P. myrtifolia* (Denancé et al., 2017). So, the phenomenon of sympatry can be deemed a crucial factor in facilitating the occurrence of these recombination events. In addition to recombination among strains of this pathogen, how they interact is of great importance.

Bacteria can display different behavioral patterns like cooperative, competitive behavior including predation. Bacterial cooperation refers to the phenomenon where individual bacteria work together in a coordinated manner for the benefit of the group. This cooperation can occur between bacteria of the same species or between different species by quorum sensing. In bacterial populations, it is typical for a signal to be continuously synthesized by individual bacteria at a low concentration in a nascent culture, and subsequently accumulate in the local environment as the density of the population increases. Upon reaching a threshold concentration, this signal molecule interacts with a receptor protein, leading to a coordinated modulation of gene expression within the bacterial community (Abisado et al., 2018).

Another microbial interaction could be exploitative in which nutritional resources are a focal point of microbial competition (Hibbing et al., 2010). These interactions can be competitive, with bacteria competing for limited resources such as nutrients and space. This also called scramble competition in which one competitor deprives another of a resource (such as a nutrient or habitable space) by depleting that resource (Hibbing et al., 2010). In a well-mixed environment to which the input of new nutrients is minimal, such as a shaking liquid bacterial culture, individuals with similar nutritional requirements, such as members of the same population, will be in competition for acquisition of these nutrients as they become depleted by the growing population (Hibbing et al., 2010).

Another way bacteria can interact is through the production of secondary metabolites. Produced during stationary phase, bacteria can be influenced by the presence of other bacterial strains, with some strains producing more or less of certain metabolites in response to competition. Some bacterial secondary metabolites have antimicrobial activity against other community members. Their interactions are known as interference competition or antibiosis and are important in the assembly and the maintenance of microbial communities (Jacobson et al., 2018).

The objective of this project is to investigate the bacterial interactions between *X. fastidiosa* by using experimental techniques to determine if their outcome is influenced by geographic location, host, and subspecies.

Materials and Method

Bacterial strains and growth conditions

Five previously tagged strains of *X. fastidiosa* from different hosts and geographic locations were used in this experiment. The origin of the strains is follows: TemeculaL was isolated in California from grapevines, WM1-1 isolated in Dahlonega Georgia from grapevine *Vitis vinifera* Mourvèdre (Parker et al., 2012), AlmaEm3 isolated from Alma Georgia from blueberries *Vaccinium* Emerald (Oliver et al., 2014), EB92-1 from Elderberry *Sambucus sp.* from Leesburg Florida (Hopkins, 2005), and BB08-1 isolated from Palatka Florida from blueberries *Vaccinium* Windsor (Donald Hopkins, Florida). The strains were grown on PWA medium amended with 10µg/ml of chloramphenicol for TemeculaL-NS1-CmR and 30µg/ml of kanamycin for WM1-1-pAX1-KmR, AlmaEM3-pAX1-KmR, EB02-1-pAX1-KmR and BB08-1-pAX1-KmR, for 7 days at 28° C and later re-streaked for use in experiments.

Co-culture experiments

Previously grown cells were scraped from PWA plates and adjusted to an $OD_{600} = 0.8$. For co-cultures an adapted version from (Navarrete et al., 2014) was carried out, a sterile 50ml Falcon tube containing a sterile glass slide inside was used to collect the biofilm produced by the cells. 25ml of PD2 media was added to each tube. For co-culturing experiments treatments included TemeculaL+WM1-1, TemeculaL+AlmaEm3, TemeculaL+EB92-1 and TemeculaL+BB08-1 to indicate what bacteria were co-cultured within the tube. To inoculate tubes, monocultures were seeded with 10µl of the bacterial suspension and for co-cultures 10µl of each strain was added to the media. The tubes were incubated for a period of 5 days at 28° C until biofilm was seen on the sides of tubes and on the glass slide. The biofilm was later aseptically scraped and suspended in

1ml of PD2 for serial dilution plating on plates containing chloramphenicol and kanamycin to distinguish between strains as shown in (Figure 1). A small aliquot of 200µl was saved from each tube for qPCR analysis.

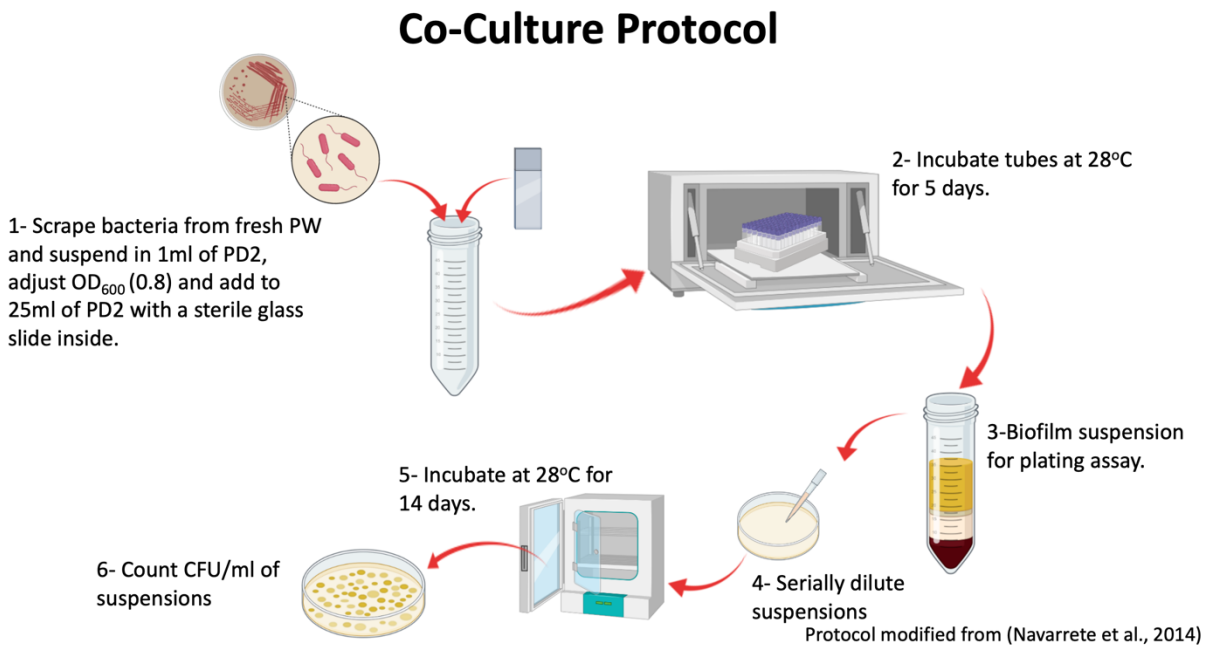

Figure 1. *in vitro* co-culture experiment for *X. fastidiosa* competition

Genome mining of bacterial secondary metabolite biosynthetic gene clusters using antiSMASH 7.0 bacterial version and BAGEL4.

Bacterial genome mining was performed using antiSMASH 7.0 a web-based tool for the identification of secondary metabolite biosynthetic gene clusters. Genomes from TemeculaL, WM1-1, AlmaEM3, EB92-1 and BB08-1 were analyzed using antiSMASH 7.0. The genomes were first downloaded from NCBI in FASTA format and uploaded for analysis. The analysis was performed using default parameters using detection strictness on "Strict mode" to predicts well-defined clusters containing any genes that code for antimicrobial compounds. The results of the antiSMASH 7.0 analysis were used to identify any biosynthetic gene clusters in the bacterial

genomes, and to predict secondary metabolites produced by these gene clusters. Using the same genome files prediction of biosynthetic gene cluster was also performed using BAGEL4. The genomes files were uploaded in the software and were analyzed under the default settings to predict clusters.

Antagonism screening by cross-streak plating and twitch motility assay

Cross-streak method.

Cross-streak method was performed as described by (Validov et al., 2006) with modification to be used for *X. fastidiosa* experiments. Strain TemeculaL was spread across Periwinkle agar (PWA) in a band approximately 1 inch wide. After 7 days at 28 °C, the plate was irradiated with UV light for 1 min and bacterial growth was scraped using a sterile disposable scraper from the agar. The plates were exposed to chloroform vapor using a soaked Whatman #4 (7.0cm) filter to kill remaining bacteria in the agar surface. Experiment was divided in two: Constitutive and Induced. Constitutive; the indicator strains WM1-1, AlmaEM3, EB92-1 and BB08-1 were previously grown separate in PWA for 7 days at 28 °C and a loopful was suspended in PD2 broth. The suspended bacteria were spot plated (20µl), and the plate was tilted to allow the bacteria to grow perpendicularly to the tester band of the previously grown TemeculaL. Induced; the strains were grown in co-culture as follows: TemeculaL+WM1-1, TemeculaL+AlmaEM3, TemeculaL+EB92-1 and TemeculaL+BB08-1 and this was used as the tester band as described previously. The indicator strains were spot plated and same procedure was carried out as the constitutive strains. Both Constitutive plates and Induced plates were grown 7 days at 28 °C.

Dual culture using twitching motility assay.

To assess antagonistic activity between closely cultured bacteria the strains were dual plated in PWA without the supplementation of Bovine Serum Albumin (BSA). This surfactant was not added to the PWA plates as it impairs twitching motility of *X. fastidiosa* (Galvani et al., 2017). Strains were grown in PWA for 7 days and later re-streaked for another 7 days and incubated at 28 °C. The bacteria were scraped and later picked up using sterile wooden toothpicks. Bacterial aggregates were placed on the PWA surface carefully right next to each other. Motility of strains was recorded for a period of 3 days using (NIS-Elements imaging Software version 3.0) with an inverted microscope (Nikon Eclipse T*i*) to visualize outward movement of the cells towards the closely plated competitor.

Bacterial growth curve

Cells were grown on PWA plates 7 days and incubated at 28 °C and later re-streaked and incubated for the same period. Cells were later suspended in PD2 broth and adjusted to an $OD_{600:}$ 0.1. Aliquots of 200µl of bacterial suspension were placed in a 96 well plate and incubated for 7 days at 28 °C. Absorbance was recorded daily at the same time using Cytation 3 Imaging Reader by BioTek.

Greenhouse experiment

Nicotiana tabacum "Petite Havana SR1", (Plant Introduction (PI552516) was used as a non-natural host of *X. fastidiosa.* Plants were grown using germination trays and seeds were placed in parallel on potting soil (Pro Mix General Usage). After 21 days 100 plantlets were placed in 4.5inch pots and watered every 2-3 days. Once plants have three true leaves (Day 27) plants were divided in groups of 10 for a total of 10 treatments. Treatments were divided by single infected

and coinfected plants. Single infected plants contained only one experimental strain and Co-infected plants contained two strains. Co-infected plant groups were: TemeculaL+WM1-1, TemeculaL+AlmaEM3, TemeculaL+EB92-1 and TemeculaL+BB08-1. Infection process was done by suspending bacteria on sterilized PBS and adjusting the cells to OD_{600} = 0.8. The suspended cells were kept in sterile 5ml tubes and used as inoculants. To allow successful infection the plants were pinpricked at the axil of the leaf and 20µl of suspended bacteria was placed on the mechanically made infection site as shown in (Figure 2). The plants were re-infected 7 days after first infection to allow better symptom development. Once symptoms of scorching started developing disease severity was recorded for a period of 9 weeks.

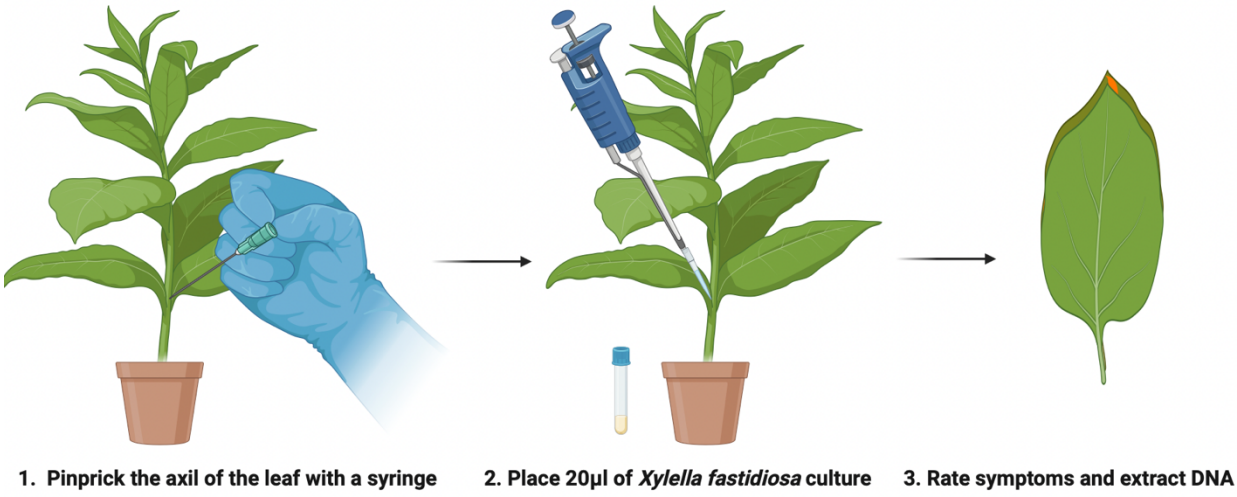

Figure 2. *Nicotiana tabacum* infection process with *X. fastidiosa* (De La Fuente et al., 2013).

Bacterial population *in planta*

Leaf petiole samples (100mg) were taken at three time points depending on development of symptoms acropetally for bacterial population. Petiole samples were later placed on 2ml tubes

and approximately 10 Zirconia beads were placed along with the petiole. The sample was macerated using a Macerator from Biospec Products for a period of two minutes until fully homogenized. DNA extraction was carried out using CTAB method and samples were later stored at -20° C.

For bacterial population quantification, primers designed from unique sequences of the strains (Chapter 2) were used. The qPCR reaction mixture was carried out using CFX96 Real-Time System using Sso Advanced Universal SYBR green supermix. For quantification of *Xylella fastidiosa* TemeculaL primer set **CmRF2: AGAAACTGCCGGAAATCGT** and **CmRR2: GGGATAGTGTTCACCCTTGTT** was used, WM1-1 primer sets for Aminoglycoside 3'-phosphotransferase **KmRF4: ATGCTTGATGGTCGGAAGAG** and **KmRR4: GTAGCGTTGCCAATGATGTTAC**, AlmaEM3 novel primers **AF9: CGCCCAAACTTGAGCATTAC** and **AR9: CATTGTTGCCGCAGATGATT**, EB92-1 **EB92F: CCCAGCCGAAGAAGTACAG** and **EB92R: TCACCTGCCTTTGGTGTATC** and last for BB08-1 **BB08F: CGTCGGATTACCCTTCCTTC** and **BB08R: GAGTTGATGGGAGCCTTCTT**. The qPCR reaction mixture included SYBR green 10µl, forward primer 1µl, reverse primer 1µl, template DNA 1µl and Nuclease free water 7µl. The qPCR reaction parameters were: 95° C for 3 minutes, denature 95° C for 30 seconds and then the annealing/extension was combined into a single step with an optimum temperature of 65° C for 45 seconds.

Statistical analysis

Colony forming units were converted into Log_{10} CFU/ml to calculate any statistically significant differences between the samples. A non-parametric test Kruskal-Wallis was performed

for comparisons and homogeneous grouping on bacterial counts, AUDPC, AUC. Statistical analysis was performed using Statistix 8 Student version. The greenhouse experiment was performed as a completely randomized design.

Results

Co-culture experiments

The serially diluted plates after 14 days at 28°C showed levels of growth for each of the co-culture combinations. Notably, TemeculaL showed prominent growth in co-culture, inhibiting the growth of the grapevine strain WM1-1 where no colonies were present in the any of the dilutions plated. TemeculaL also limited the growth of other strains from subspecies *multiplex* (AlmaEm3, BB08-1) and the avirulent strain EB92-1, suggesting that it may have a competitive advantage over the other strains while in co-cultures as shown in (Figure 3).

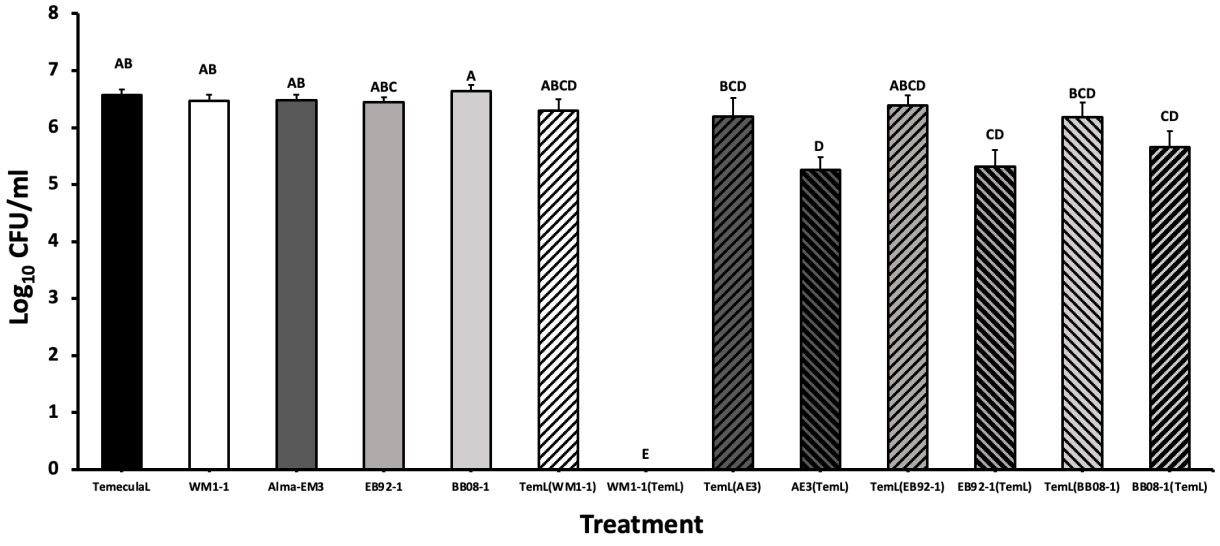

Figure 3. Monoculture and co-culture results from biofilm phase after 14 days of incubation on PWA with their respective antibiotics. Different letters indicate statistical significance using

Kruskal Wallis non-parametric test. Statistical analysis was carried out using Statistix 8 Student version. Data represented is from one experiment that was repeated three times independently.

Data from the planktonic phase was not recorded due to some plates including controls not showing growth. Total counts were not considered as this analysis was meant to be differential.

Detection of biosynthetic gene clusters using antiSMASH 7.0 and BAGEL4

Genomic analysis with antiSMASH to identify any possible secondary metabolites present within the genome of *X. fastidiosa* was performed. No secondary metabolite of antagonistic interest was predicted using this software. Using stringent analysis, only a protective pigment related to carotenoids was identified as arylpolyene on all five experimental strains on different positions shown in (Figure 4). The same analysis was performed using the Temecula1 reference genome which is a fully sequenced and annotated and the pipeline detected the same biosynthetic gene cluster with Locus tag PD_1142 and annotated as 3-oxo acyl- (ACP) synthase (data not shown). Same analysis was carried out with BAGEL4. The software detected on all 5 experimental strains the cluster which codes for a Colicin V as shown in (Figure 5). Colicin V is a well described bacteriocin in *Escherichia coli* that kills closely related species. It has been reported that *X. fastidiosa* has a Colicin V like precursor that is among the CDS most frequently expressed by this pathogen, even under standard growth conditions in periwinkle medium (Zaini et al., 2008). This suggests that this bacteriocin is a common weapon employed by this pathogen, possibly to counterbalance its slow growth and to allow efficient competition with other microorganisms within the xylem and insect foregut (Zaini et al., 2008). Using the Temecula1 reference genome all sequences relating to Colicin V machinery were present, *cvaC* (PD_0215), *cvaA* (PD_0496),

cvaB (PD_0499) but the *cvi* which confers immunity is not annotated in the genome. An analog sequence from *Escherichia coli* (Q841V5) has been reported to match a putative *cvi* in *X. fastidiosa* 9a5c (XF0216) (Pashalidis et al., 2005). The reported analog matches with the locus tag (PD_0214) on the Temecula1 genome. The sequences were found on different positions within the genome as also described by (Chen et al., 2020) different than in *E. coli* that are arranged in an operon in the pColV plasmid (Gilson et al., 1987).

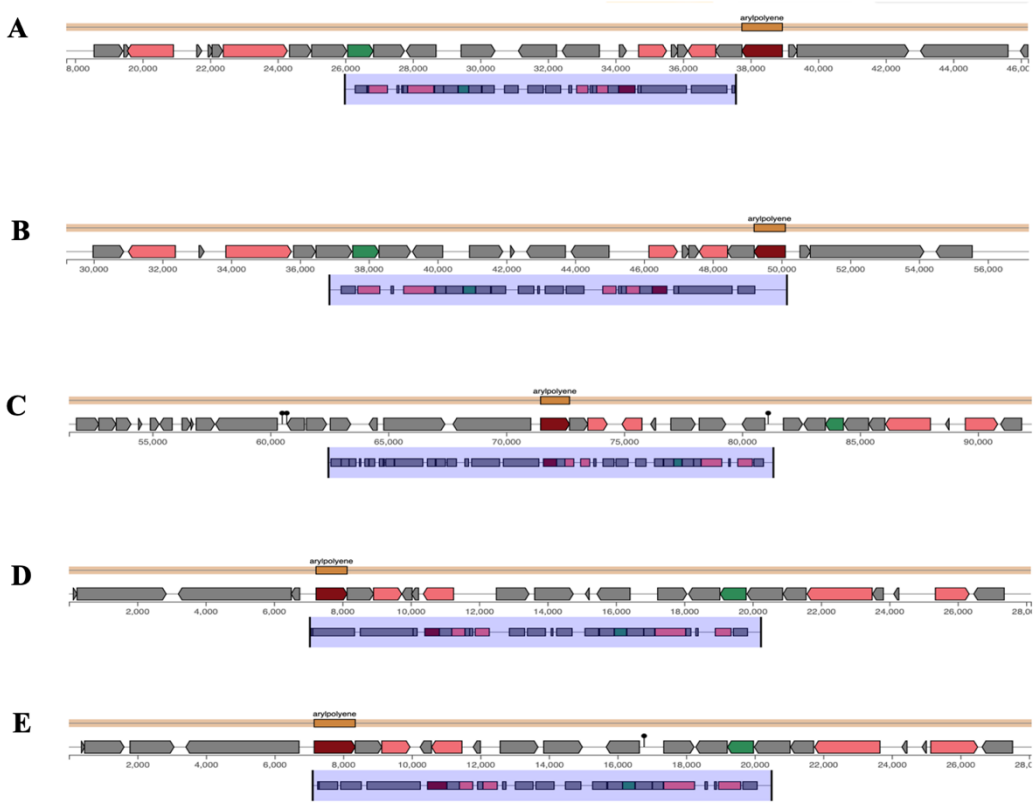

Figure 4. Biosynthetic Gene Cluster detected by antiSMASH bacterial version. (A) TemeculaL, (B) WM1-1, (C) AlmaEM3, (D) EB92-1, (E) BB08-1. The pipeline only detected arylpolyene with and annotation of 3-oxoacyl (ACP) synthase and a locus tag in the Temecula1 genome PD_1142.

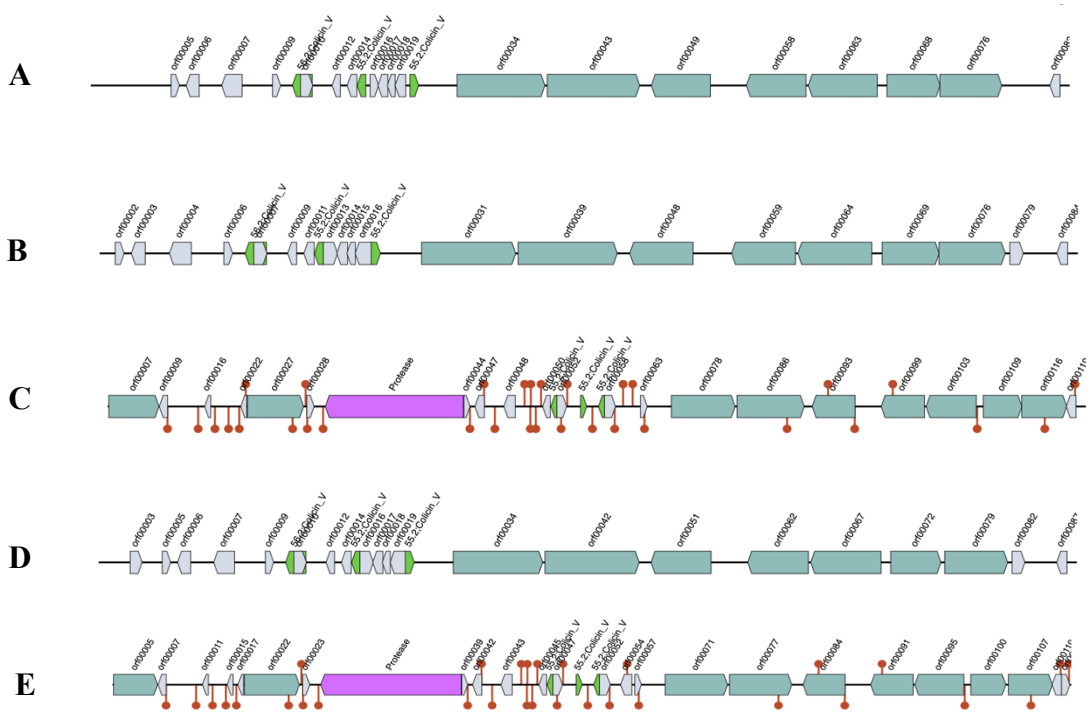

Figure 5. Biosynthetic Gene Cluster detected by BAGEL4. (A) TemeculaL, (B) WM1-1, (C) AlmaEM3, (D) EB92-1, (E) BB08-1.

Blast alignments of the putative *cvi* reported by (Pashalidis et al., 2005) showed 95% identity with the *subsps*. *fastidiosa* strains used in this experiment and 96% identity with the *multiplex* strains. Meaning that the remaining gene for the bacteriocin machinery could possibly be present.

Antagonism screening

The constitutive and induced cross-streaked plates were examined for the presence of an inhibition halo between the tester and indicator strains. However, no visible inhibition halo was observed in either the constitutive or induced plates. These results suggest that the tested strains did not produce any inhibitory substances or that the tested strains were not susceptible to

inhibition by their neighboring strains under the given experimental conditions as shown in (Figure 6).

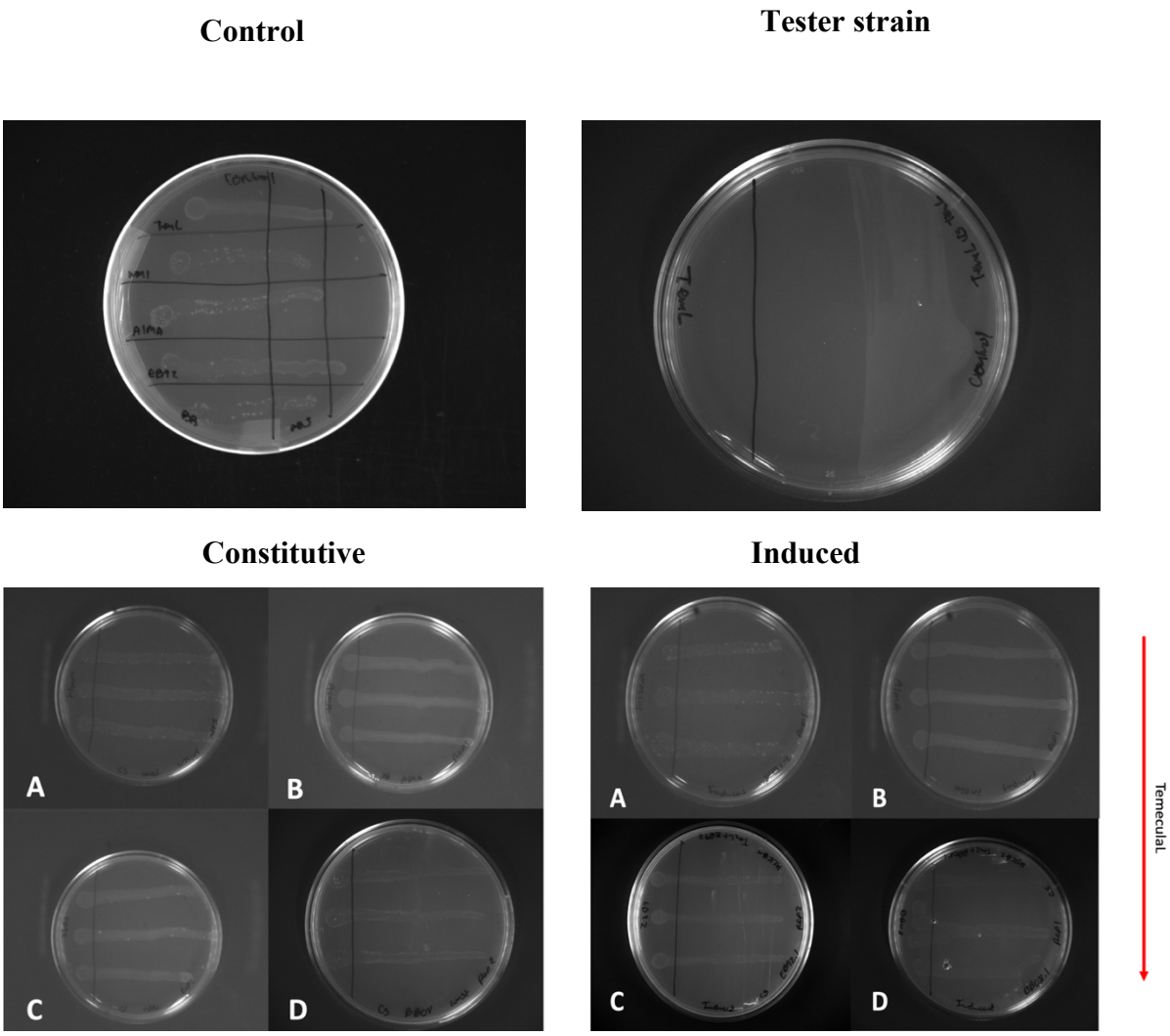

Figure 6. Controls and tester streak is the top image. Cross-streak "Constitutive" Left. (A) WM1-1, (B) AlmaEM3, (C) EB92-1, (D) BB08-1. "Induced" Right. (A) WM1-1, (B) AlmaEM3, (C) EB92-1, (D) BB08-1. Represents one experimental replicate; similar results were observed on all repetitions (n=3).

The twitching motility assay was conducted to evaluate the potential inhibitory effect of the tester strains against the indicator strains when in proximity. The assay was performed for a period of 3 days due to the growth of bacterial aggregates toward each other which resulted in the loss of visible fringes. Notably, no inhibition halo was observed within the fringes during the experimental period, indicating a lack of inhibitory effect by the tester strains against the indicator strains as shown in (Figure 7).

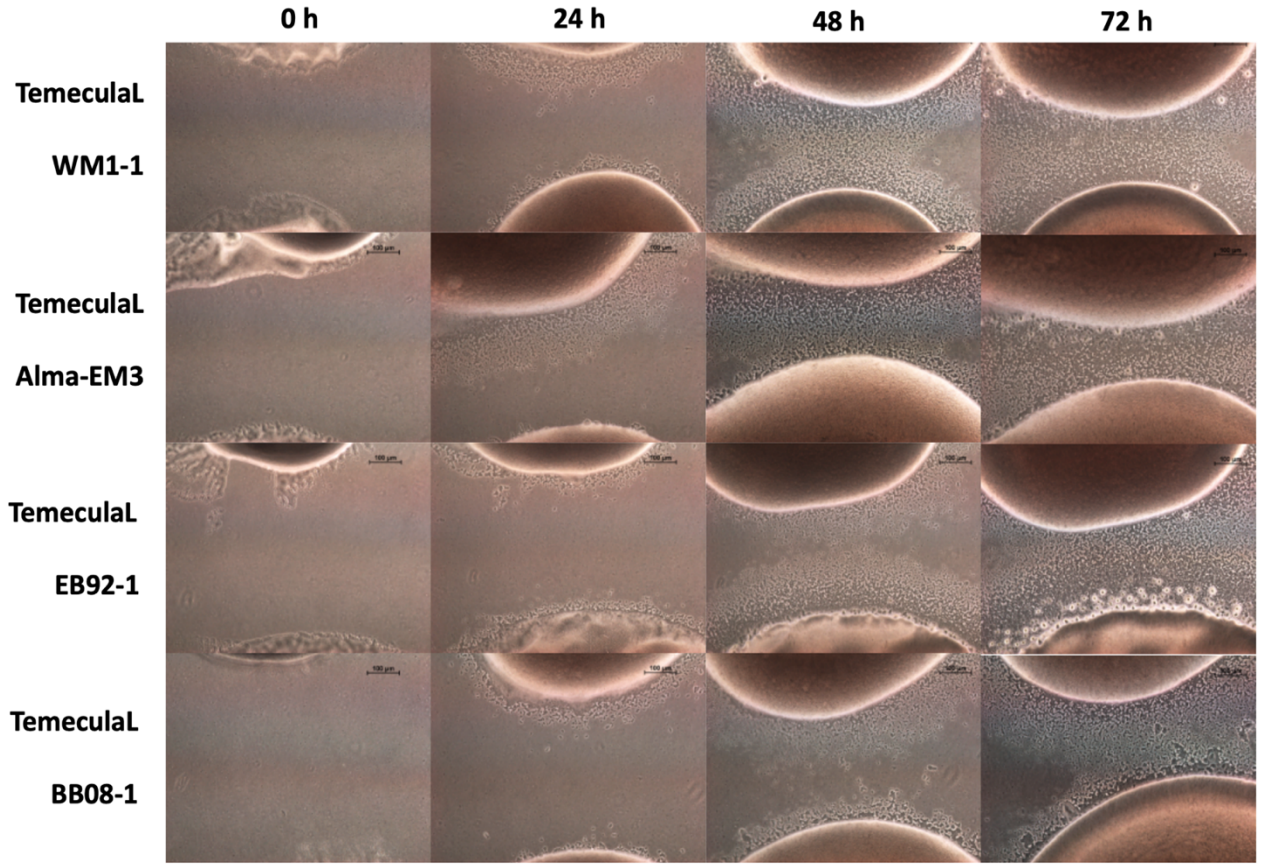

Figure 7. Fringe morphology of *X. fastidiosa* strains under competition, bacterial twitching can be seen moving outward from the bacterial aggregate coming in contact with the neighboring cells placed on the PWA plate.

Growth curve

To further explore the individual behavior of the strains, a growth curve was performed over a period of 7 days. As shown in (Figure 8), TemeculaL exhibited a faster growth rate, reaching a peak on day 5, compared to the other strains. EB92-1 showed a high peak on day 6. Area under the curve showed statistical significance of TemeculaL from strains WM1-1, AlmaEM3 and BB08-1 and no statistical significance towards EB92-1.

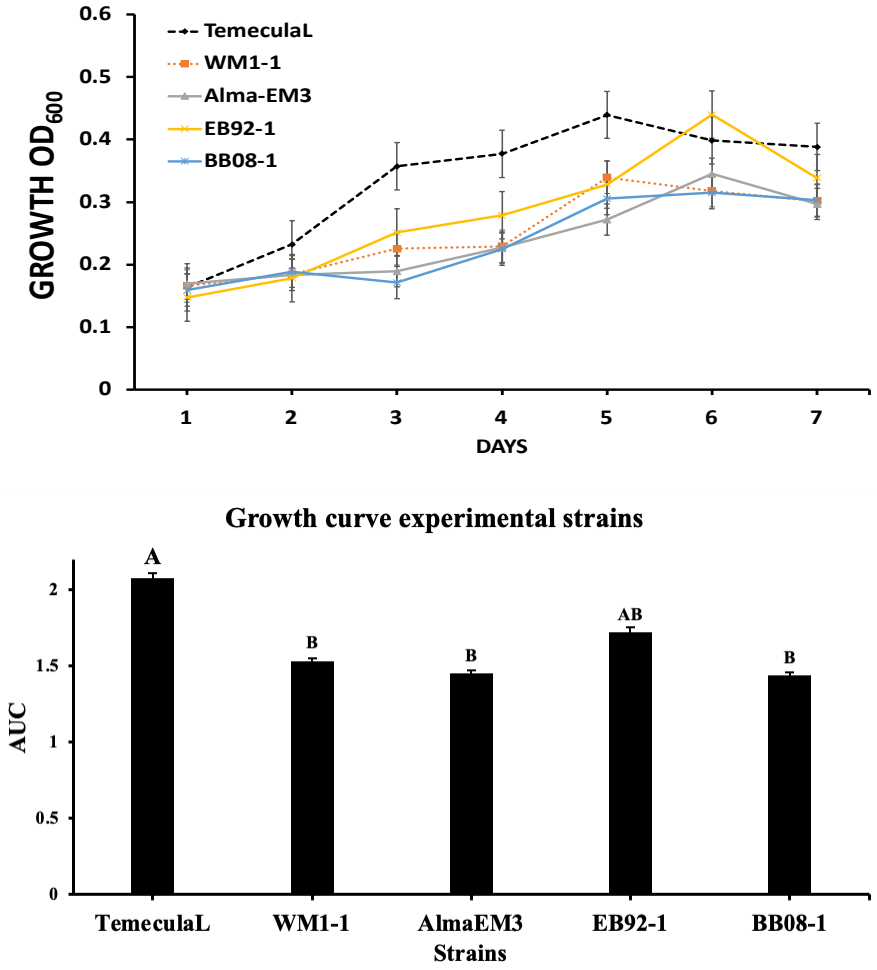

Figure 8. Growth curve of all 5 experimental strains on PD2 liquid media. Error bars represent standard error. Experiment was performed one time in triplicates.

Greenhouse Experiment

Symptoms of leaf scorching first appeared 6 weeks after inoculation as shown (Figure 10). Upon observing the onset of symptoms, the severity of the disease was monitored on a weekly basis and recorded for analysis of disease progression. Scorching severity was rated on a scale from 1-5, which provided information on the extent of scorching coverage on the affected leaves in terms of percentage as shown in (Table 1). Throughout the 9-week experimental period, all treatments showed a constant rate of severity in which the acropetally visible symptoms progressed slowly as shown in (Figure 11). However, the group of plants co-infected with TemeculaL+BB08-1 exhibited more severe symptoms, reaching a disease severity of 29.14% compared to other treatments. The AUDPC analysis confirmed the statistical significance of this group towards treatment group EB92-1 and TemeculaL+EB92-1, indicating that symptom development worsened when tobacco plants were infected with both strains. Conversely, plants infected with the avirulent strain EB92-1 displayed some scorching patterns but no severe symptoms, with a disease severity of 5.14%. However, co-inoculation of EB92-1 with TemeculaL resulted in a reduction of scorching and a disease severity of 3.72%, suggesting that EB92-1 was effective in controlling disease progression in plants, as previously reported as a potential biocontrol strain as shown in (Figure 9).

Table. 1 Leaf scorching scale

Scale	Percentage
0	0%
1	10-25%
2	25-50%
3	50-75%
4	75-100%

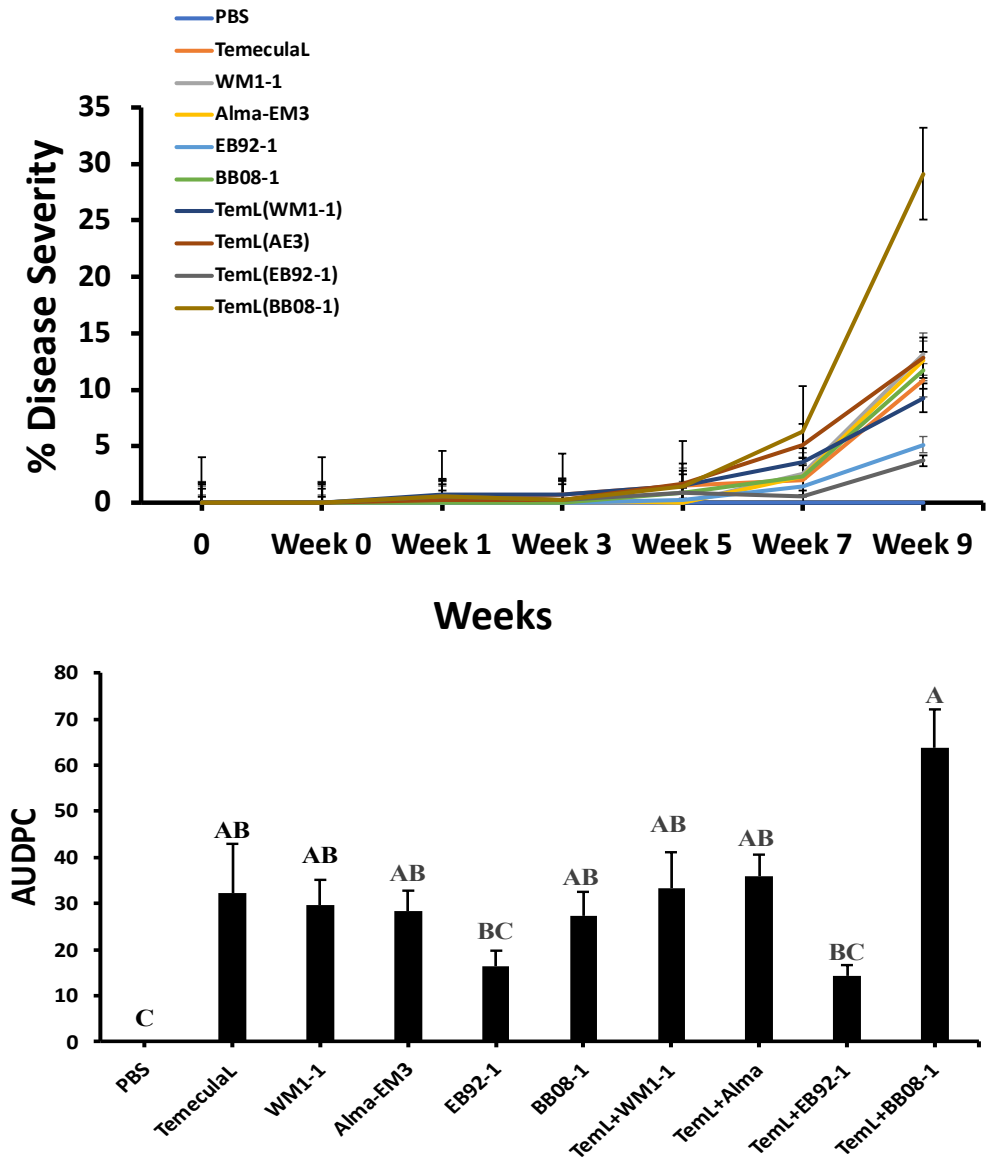

Figure 9. Disease severity curve of *Nicotiana tabacum* SR1 Petite Havana after 9 weeks post symptom appearance. AUDPC of infected plants after 9 weeks post symptom appearance. Bars represent mean and error bars represent standard error. Different letters indicate statistical significance using Kruskal Wallis non-parametric test. Statistical analysis was carried out using Statistix 8 Student version.

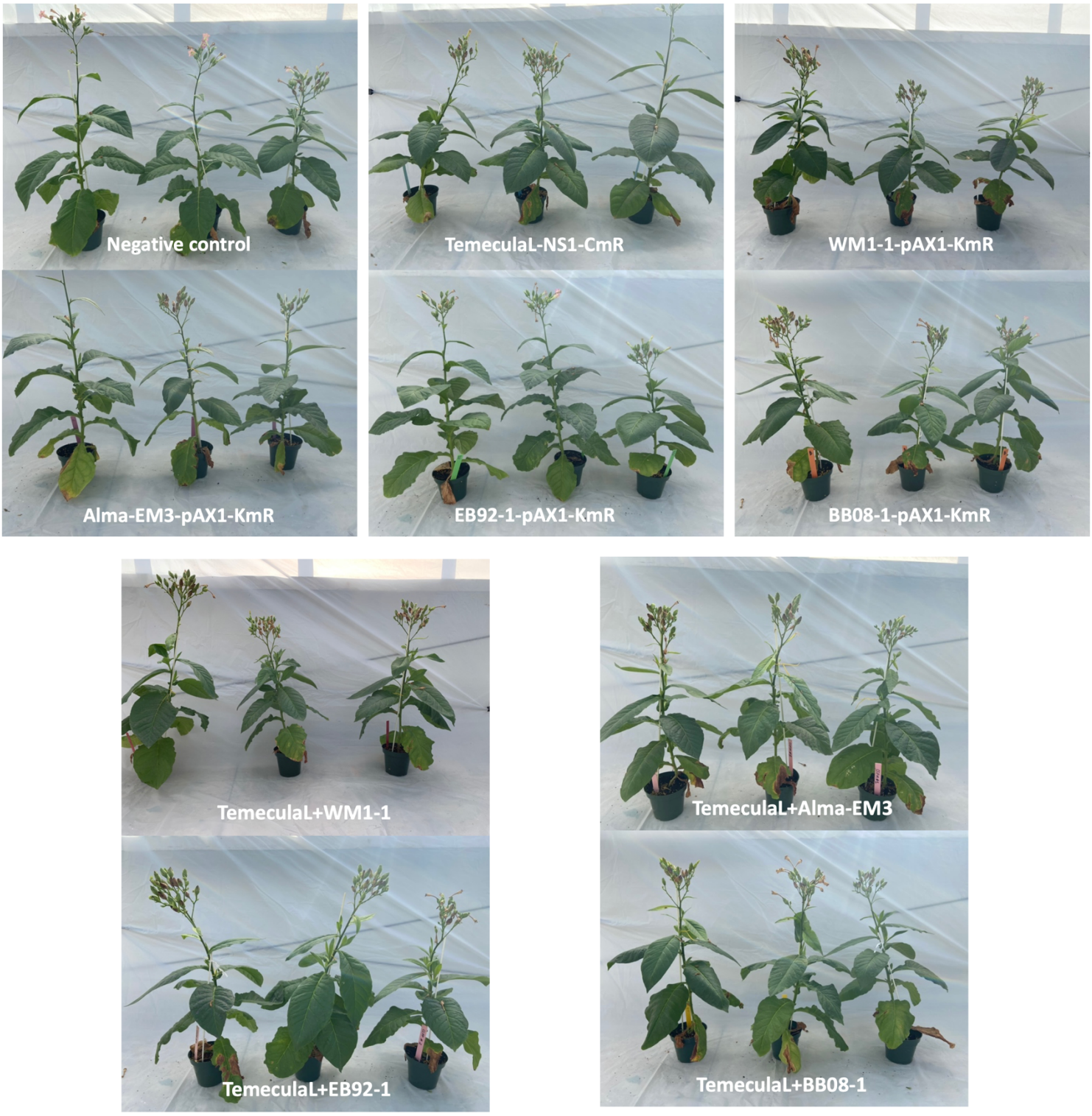

Figure 10. Tobacco plants showing first signs of scorching on basal leaves 6 weeks post inoculation.

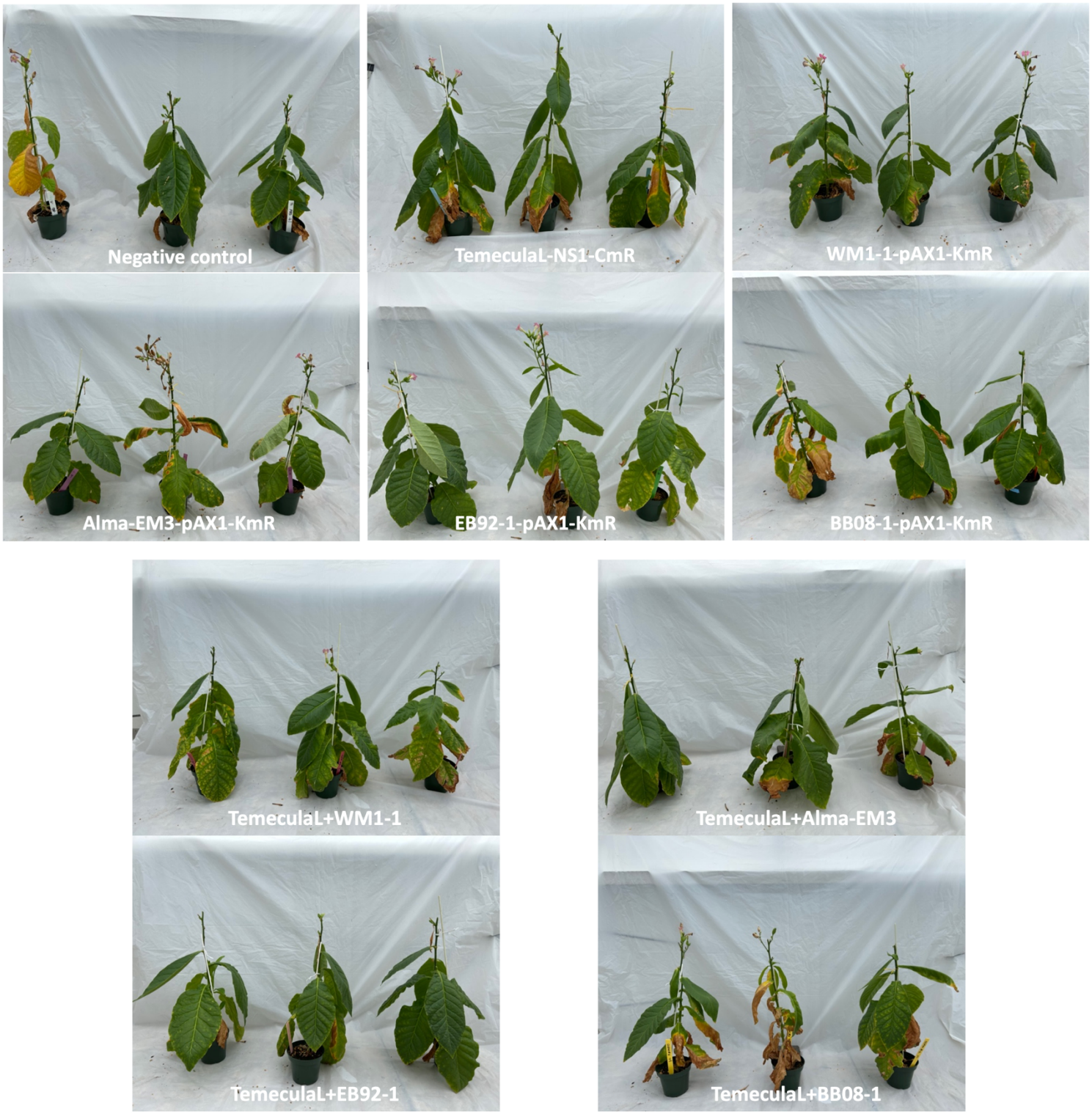

Figure 11. Tobacco plants showing signs of scorching acropetally 9 weeks post infection.

Bacterial population *in planta*

The quantification of bacterial population within plants was impeded by the presence of noisy data observed in the qPCR analysis. The assay used a sensitive intercalating dye, SYBR green, that binds to double-stranded DNA and fluoresces during amplicon synthesis. The inconsistent amplification curves and multiple small peaks in the samples suggest non-specific amplifications as shown in (Figures 11 and 12). The cause of this issue may be attributed to the lack of specificity of the dye used for this analysis. SYBR green binds to any dsDNA including target and non-target (Giulietti et al., 2001). To overcome this limitation, a possible approach is to design primer probes specific to this experimental setup. The presence of plant genetic material may have interfered with the assay, but specific primer probes would only bind to a targeted fragment of the bacterial DNA. This enables bacterial quantification within the plant. Protocols regarding *X. fastidiosa* primers like HL5 and HL6 (Francis et al., 2006), subspecies specific primers developed by (Dupas et al., 2019) and primers for the detection of the Citrus Variegated Chlorosis *X. fastidiosa* subsp. *pauca* developed by (Ouyang et al., 2013) have a probe designed for the assay. Since the qPCR primers are validated, the probes can be designed using the same primer design software Primer Quest with the custom parameter design by uploading the template target sequence along with the forward and reverse primers.

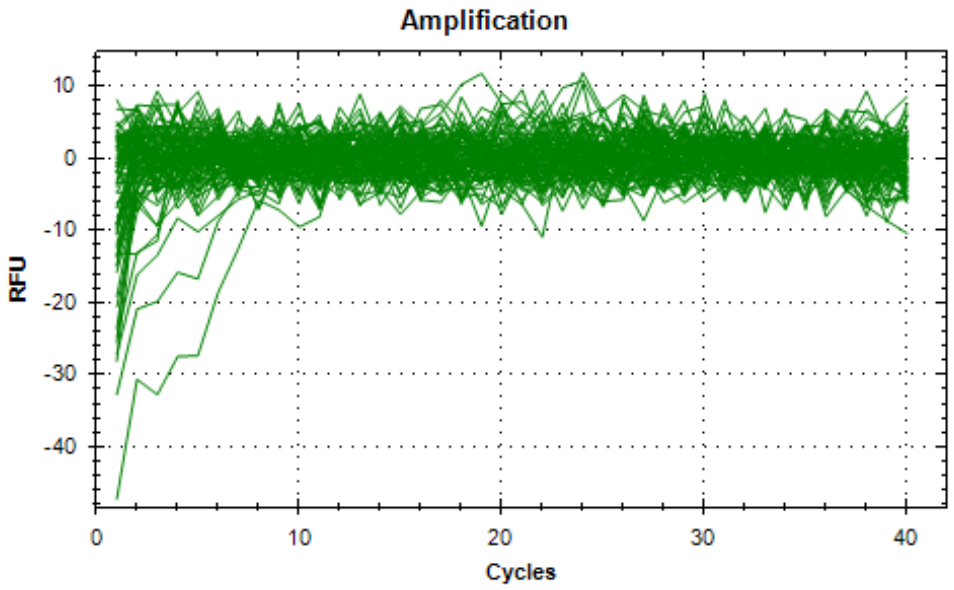

Figure 12. Noisy data of *in planta* bacterial quantification using novel primers coupled with SYBRgreen.

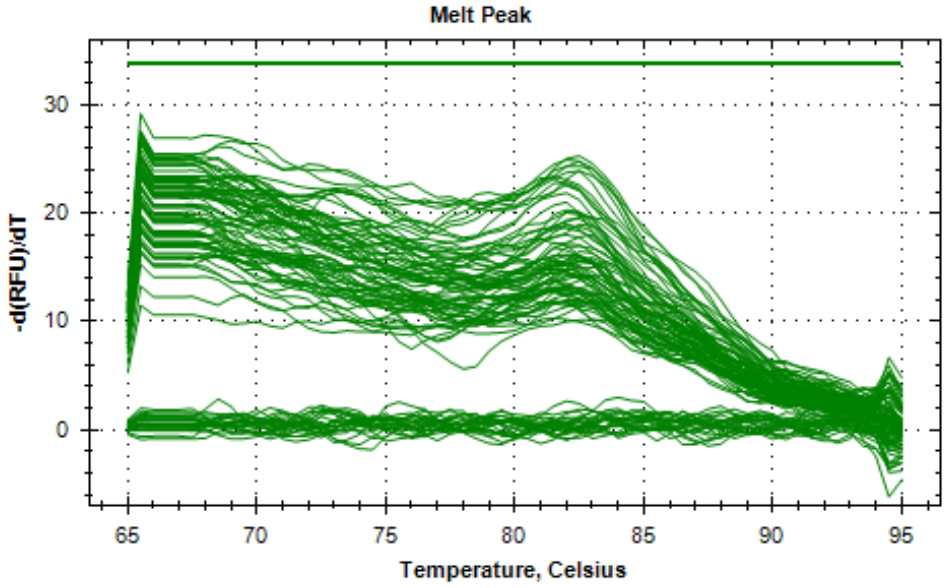

Figure 13. Melt peaks of *in planta* bacterial quantification using novel primers coupled with SYBRgreen.

Discussion

Understanding the factors that contribute to bacterial competition can provide valuable insights into the ecology and evolution of bacterial communities. The complex dynamics of bacterial competition and its implications in different contexts are catalyst to the evolution of bacterial communities. Our results from *in vitro* studies using TemeculaL in co-culture with other strains showed a clear competitive advantage over the other strains. The growth of WM1-1 strain was completely inhibited in co-culture with TemeculaL as indicated by the absence of colonies in all dilutions after 14 days of incubation. Additionally, the growth of the other strains from subsps. *multiplex* (AlmaEm3, BB08-1) and the avirulent strain EB92-1 (subsps. *fastidiosa*) was also limited, indicating that TemeculaL may be exerting an inhibitory effect on their growth. Complete inhibition of growth was mainly seen with strain from the same subsps. *fastidiosa* that shared the same host which is grapevine. We can infer that coming from the same host these strains can possibly have some metabolic similarity that can make them compete for space and available nutrient. To assess if metabolic profiles of *X. fastidiosa* correlates with antagonism, the BIOLOG systems a described by (Gerlin et al., 2020) could be a useful tool. It has been suggested that metabolic similarities correlate with antagonism of closely related species as described by (Russell et al., 2017). The study found a strong correlation between antagonism and phylogenetic distance, with closely related species being more likely to show antagonism. They also found that antagonism was more likely between metabolically similar strains, and this was true regardless of whether the strains were sympatric or allopatric. Our study only used five strains and more combinations are needed to conclude what has been published and what looked to be evident from this experiment. The observations of this experiment suggest that TemeculaL may have a distinct

advantage in the competitive environment, which may be attributed to its ability to better utilize available resources, or interference competition. Bacteria that are closely related and that occupy the same niche has also been speculated to engage in competition (Segura- Munoz et al., 2020). Given that *X. fastidiosa* ecological niche is the xylem vessels and mouthparts of the vector (Landa et al., 2022), these restricted environments with poor nutrients can lead to active competition. The possibility of this pathogen to employ a competitive behavior to colonize and ward off the competition this would be the first report of interspecies and intraspecies *in vitro* competition of *X. fastidiosa.*

Given the absence of growth of WM1-1 and the reduction of other strains in co-culture with TemeculaL, the possibility of antagonism between the strains arose, leading this research to consider the presence an antimicrobial compound as a possible explanation. Computational analysis of the bacterial genomes using antiSMASH 7.0 and BAGEL4 to predict any biosynthetic gene cluster that could show sequences for antagonistic molecules like bacteriocins was performed. Using genome files from the experimental strains antiSMASH 7.0 only detected the presence of arylpolyene. These compounds are covalently attached in the gram-negative outer membrane and have a predicted function as protective agents against oxidative stress (Goel et al., 2002, Schöner et al., 2016). But no published data on this specific secondary metabolite having antagonistic activity against closely related bacteria has been reported. Meanwhile BAGEL4 predicted in all 5 strains the presence of Colicin V. Previous studies have shown experimentally that *X. fastidiosa* expresses a gene annotated as *cvaC*. This gene is highly significant because it encodes for a bacteriocin called Colicin V, which is typically found in *Enterobacteriaceae* (Gérard et al., 2005). The Colicin V has been demonstrated in *E. coli* to attack and kill closely related

species. In the reference genome of *X. fastidiosa* Temecula1 the sequence appears annotated as a Colicin V precursor. The locus tags of the group of genes important for the synthesis and transport of this possible bacteriocin are *cvaC* (PD_0215), *cvaA* (PD_0496), *cvaB* (PD_0499) with *cvi* which give the bacteria immunity against its own bacteriocin, a homolog to *E. coli* has been reported *cvi (PD_0214).* The *cvi* gene encodes the cognate immunity protein of 78 residues, with two transmembrane helices, which alone is sufficient to fully protect a cell from the bactericidal activity of Colicin V (Gérard et al., 2005). No present evidence has been shown of this gene being expressed under competitive conditions with other *X. fastidiosa* strains, only expressed *in planta* (Souza et al., 2015), low and high iron concentrations (Zaini et al., 2008) and under calcium exposure (Chen et al., 2019). The Colicin V clusters were predicted thus confirming that this bacterium has the entire machinery for bacteriocin production. The experimental trials did not demonstrate the presence of a halo in the cross-streak method and dual plate assay using twitch motility. This suggests that the tested strains did not exhibit interference competition behavior towards each other under the tested conditions either due to stability of the bacteriocin or low concentration due to slow growth. Another protocol regarding the supernatant of these cultures would have to be tested to determine if *X. fastidiosa* engages in interference competition with other *X. fastidiosa*, in which one competitor actively harms the other (Hibbing et al., 2010). No reports have been published of *X. fastidiosa* expressing this gene when in contact with other strains. A growth curve of the experimental strains was performed to determine the growth rate of each experimental strain that could show a possible advantage in fitness. This resulted in the strain TemeculaL having a high peak in absorbance on day 5 when compared to the remaining strains WM1-1, AlmaEm3, EB92-1 and BB08-1. Meaning that this strain outcompeted the others in communal media for possible nutrient resources. A limitation to this approach is that it doesn't

simulate the environment of a co-culture and metabolic profiling would be a better option. The findings of this study may have implications for the understanding of bacterial interactions suggesting that other mechanisms, such as nutrient competition or interference competition, may have played a role in the observed competitive interactions *in vitro*.

Understanding the behavior and interactions of microorganisms in natural environments is crucial. *In vitro* experiments allow for controlled and reproducible conditions, but they cannot fully capture the complex interactions between the pathogen and the plant in its natural environment. Following the same experimental set up as *in vitro* the same bacterial mixtures were used but using *Nicotiana tabacum* SR1 Petite Havana, a non-natural host for the grapevine, blueberry and elderberry strains used in this experiment. As a result of *X. fastidiosa* infection, typical marginal scorching was present 6 weeks post inoculation on the basal leaves moving acropetally by 9 weeks. The treatment group of the co-infected plant with TemeculaL+BB08-1 showed statistical significance with a higher disease severity of 29.14% and AUDPC 69.14 when compared to the other treatments. This experimental group exhibited more severe symptoms of interveinal chlorosis followed by necrosis of the basal and top leaves. The treatment plants infected with EB92-1, an elderberry strain isolated from Florida showed some marginal scorching symptoms with disease severity of 5.14% and AUDPC 16.34 values. This avirulent strain missing genes encoding pathogenicity effectors and has been reported to be a biocontrol strain against Pierce's Disease (PD) for up to 4 years (Zhang et al., 2015, Hopkins, 2005). Similar results were shown in the treatment plants co-infected with TemeculaL+EB92-1 in which over the course of 9 weeks the symptom development was seen in some leaves. This group showed the lowest disease severity percentage of 3.72% with AUDPC 14.20 and was not statistically significant from its single

infected control, suggesting that plants infected with this biocontrol strain remained healthy through the entirety of the trial. Disease severity percentage remained low during the first few weeks of the experiment which was during the winter break. Simultaneously petiole samples of the infected plants were being collected as symptoms were developing for bacterial quantification with qPCR, but data from the reactions indicated the primers amplifying multiple nontargets from possible plant material. The assay was carried out using SYBR green, but this didn't allow for the quantification of these bacteria *in planta* as thus the data was too noisy.

In conclusion, understanding bacterial competition and its implications in different contexts can provide valuable insights into the ecology and evolution of *X. fastidiosa.*

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