

UTILIZING UVR PROTECTANTS TO OPTIMIZE BACTERIOPHAGE FOR FIRE BLIGHT
MANAGEMENT

By

Madison Dobbins

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ABSTRACT

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Erwinia amylovora is a devastating bacterial pathogen that is the causal agent of the pome fruit disease fire blight. For plant diseases, alternate biological controls, such as bacteriophage, are now the subject of many research studies as a potential substitute for the use of antibiotics. In several *in vitro* studies, bacteriophage have been shown to reduce the survival of *E. amylovora*; however, for field application, protection from degradation by ultraviolet radiation (UVR) is required. We examined if the addition of peptone or carrot juice, both highly pigmented liquids, and kaolinite clay could protect individual *E. amylovora* bacteriophage from inactivation by UVR. We studied the effects of these potential protectants to bacteriophage survival following dosing with UVR wavelengths in the UVA, UVB, and UVC range. We found that additives of either peptone or kaolinite clay acted as UV-protectants. Greater concentrations of both the peptone and kaolinite clay demonstrated greater protective qualities against UVR. In a field study, commercial bacteriophage cocktails such as Agriphage, Firequencher A and B, cocktails of *E. Amylovora* specific bacteriophage, were also used to demonstrate the protective qualities of additives, as well as the potential for bacteriophage to be used more regularly as a biological control agent against *E. amylovora*. These field studies identified three separate bacteriophage - the Agriphage cocktail (Certis and Omnylytics), $\Phi 31$ -3 and $\Phi 21$ -4 - as potential protectants against the occurrence of *E. amylovora*.

This thesis is dedicated to Lucy, the coolest cat I know.

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CHAPTER ONE:

THE BACTERIAL PATHOGEN, FIRE BLIGHT'S, IMPACT ON THE FRUIT TREE INDUSTRY AND THE POTENTIAL FOR ITS MANAGEMENT STRATEGIES

Fire blight is a devastating disease and is caused by the gram-negative bacterial pathogen *Erwinia amylovora*. Fire blight is known to be one of the most aggressive and threatening diseases to pome fruits, specifically apples (*Malus* sp.) and pear (*Pyrus* sp.), for over 100 years (Baker, 1971). The invasive lifestyle of fire blight can cause infection on rosaceous plants, allowing for the spread and infection of this pathogen to occur on nearly every continent. Fire blight was first identified in New York state in the Hudson Valley by William Denning in 1780, where he first recorded the pathogen creating cankers, a visible cracking and opening in the bark (Din et al., 2007), in pear trees and noted the spread of symptoms from the pathogen throughout the orchard. The movement of the *E. amylovora* bacterium reached the west coast of the United States by 1873, 93 years after its discovery in New York. By the 1950s, fire blight had spread internationally to Europe, less than 100 years after its initial discovery (Baker, 1971; Steiner, 2000)

IMPORTANCE OF APPLES IN THE UNITED STATES

Apples are a high-value crop that are frequently imported and exported around the world. In 2018, the United States produced 272 million bushels of apples, 25 million bushels coming from Michigan alone (USDA-NASS, 2019) While in 2016, 61% of the total apple production produced in the United States was grown in Washington, 10% in New York, and another 9% in Michigan, making these the top three apple-producing states in America (Schmit et al., 2018). Overall, 85 million metric tons of apples were produced across the world in 2015 (Schmit et al.,

2018) In the same year, the demand for fresh market apples rose to 715 million pounds, making apples a highly desired crop (Schmit et al., 2018). With apples being such a highly produced and highly desired crop, the prevention and control of fire blight is detrimental to the livelihood of producers. The best way to prevent an infection and a fire blight outbreak from occurring is to further understand how the *E. amylovora* bacterium spreads and infects.

IMPORTANCE AND IMPACT OF FIRE BLIGHT IN THE FRUIT TREE PRODUCTION COMMUNITY

Fire blight is not secluded to the United States, it has traveled worldwide, causing economic losses wherever fruit trees are grown. *E. amylovora* infects apples and pears, some cultivars have been found to be more susceptible to fire blight infection than other cultivars (Breth et al., 2006). The most popular cultivars of apples that are grown around the world are Red and Golden Delicious. However, many new varieties are being developed and are diversifying the apple industry (Schmit et al., 2018). With the diversity of cultivars there also comes speculation of some cultivars being more or less susceptible to fire blight. Susceptibility among cultivars to fire blight infection is due to specific genes and early signaling responses (Baldo et al., 2010). In 2006, more than 51% of the trees planted in New York were fire blight susceptible cultivars (Breth et al., 2006).

DISEASE CYCLE AND EPIDEMIOLOGY OF FIRE BLIGHT

The disease cycle of fire blight begins with the initial infection through either floral stigma or an existing wound (S. V. Beer, 1979; Piqué et al., 2015). Existing wounds can be caused by weather-related or pruning damage. The infection of *E. amylovora* into the nectary of the flower is the preferred mode of infection for this pathogen. However, before entering the

nectary, *E. amylovora* must grow to high populations on the stigmas of the flower (Schroth et al., 1974). Once the bacteria infect, blossom blight, one of the first visible symptoms of fire blight, becomes visible. Blossom blight appears as necrotic tissue that has spread through the entirety of the flower. Once the petals have fallen, the flower cluster will remain on the tree, and the tissue will darken with blight. Once this level of degradation has occurred from the pathogen, the fruit production from this cluster is no longer viable. The death of the flower has eliminated all fruit production of that flower and, in large quantities, can have a significant impact on the fruit production industry (Steiner, 2000).

The expansion of blossom blight further into the stem can be recognized as shoot blight. Shoot blight is recognizable as the blighted tissue that has expanded further past the blossom blight onto the stem. Generally, shoot blight can be identified as a "shepherd's crook" as the shoot tip will bend over to appear as a crook. This stage of development signifies the systemic spread of *E. amylovora* and, which will continue to migrate via the xylem cortical parenchyma tissue into the trunk of the tree if left unmanaged. Once the bacterium has reached the trunk of the tree, cankers can develop. Cankers are the overcapacity of bacterial cells, causing a crack, or burst in the trunk's external bark. Cankers are an ideal location for ooze, sap like droplets excreted from wounds or openings of the tree, comprised of Amylovoran bacteria and exopolysaccharides (EPS) (Slack & Sundin, 2017), to emerge and further the spread of fire blight through the amylovoran found in ooze. All areas of infected tissue, such as cankers, are capable of producing ooze (Steiner, 2000).

Ooze plays a significant role in the infection and spread of fire blight, due to its presence on trees and the population of bacteria present within a single ooze droplet. Ooze has been identified to most likely be present seeping out of cankers, and wounds within two weeks of

flower bloom occurring (Beer & Norelli, 1977). On average, a single ooze droplet contains 108 CFU/ μ l of amylovoran, along with high levels of EPS. Exopolysaccharides are beneficial to the ooze as they protect the amylovoran cells as well as give the ooze its viscous structure. Different levels of amylovoran give the ooze varying shades of colors, such as white, yellow, and brown. Generally speaking, the darker the color, the greater amount of amylovoran. The bacterium's presence as ooze can be seen seeping from infected tissue and wounds in droplets. Ooze droplets can range in size, being found as small as 1 μ l, all the way up to 20 μ l (Slack et al., 2017). With ooze being the most critical component in the spread of fire blight, it can commonly be found near flowering clusters or exposed wounds and cankers. While ooze may be the primary vessel for *E. amylovora*, further spread cannot happen without the necessary water droplets and vectoring insects.

The spread of bacteria can be completed by vectors such as flies, ants, and bees in some speculations (Beer, 1979). The insect vectors commonly will come into contact with the ooze while pollination and will thusly become a vector for the pathogen. There is a controversy over whether bees are to be considered a vector or not; Vanneste (2000), states that honeybees do not have an attraction to the ooze, and do not act as a primary dispersal agent (Hortresearch, 1996; Vanneste, 2000). Recently, the potential for bees working as vectors was questioned (Cellini et al., 2019). The main factor in question was whether *E. amylovora* changed the overall scent of the infected flowers and if that had an impact on whether or not honeybees would find infected flowers more attractive to pollinate? In conclusion it was determined that the scent change encouraged the honeybees to be more attracted to uninoculated flowers, thusly, bees should be considered as a secondary vector (Cellini et al., 2019). Furthermore, to investigate bees as vectors, pollen inserts were attached to hives, forcing bees to walk through *Erwinia* bacterium as

they left the hive. At the end of their study, it was concluded that bees vectored 20 blossoms on average per hour that the bee is actively foraging (Johnson et al., 1993). Additionally, other insects had been observed for their abilities to vector *E. amylovora*, precisely the length of time specific insects can harbor the active bacteria. Aphids were found to have one of the longest survival time for *Erwinia* at 12 days, and the green lacewing still proving to be very impressive at five days of survival (Hildebrand et al., 2000). The most common vectors responsible for the spread of *E. amylovora* are insects, birds, and humans. All having the ability to come into contact with the bacteria and spread it unknowingly (Beer, 1979).

ENVIRONMENTAL FACTORS EFFECTING FIRE BLIGHT INFECTION

The infection process of *E. amylovora* begins when the pathogen is introduced into a new orchard or area; Infected trees serve as an inoculum source for the spread of the pathogen. For *E. amylovora* to create an infection, temperatures must be equal or greater to 18.3C for a minimum of 110 hours with an average daily temperature of at least 15.6C. In addition to temperatures, appropriate amounts of water (> 0.25 mm of dew), allows for the bacteria to become mobile and spread (Steiner, 1990). Weather plays a significant role in the acceleration of the growth and spread of *E. amylovora* in creating causing blossom blight. If conditions are ideal with appropriate wetness and warm temperatures, a blossom blight epidemic can occur suddenly, the most vulnerable time for a fire blight infection to occur is within the first three weeks of the flowers losing their petals (Johnson, 2000). While if wetness does not happen or the temperatures do not rise and stay constant, *E. amylovora* will lie dormant, and it can take upwards of a month for blossom blight infection to occur (Steiner, 2000).

CURRENT MANAGEMENT METHODS OF FIRE BLIGHT

If properly maintained, orchards can flourish for several decades; however, this cannot be done without integrated pest control, using chemical, physical, cultural, and biological tactics. The fire blight infection has a menagerie of controls that work separately in their modes of action but can be combined for optimal disease control and prevention. The control methods have been successful in reducing the total number of infections when applied correctly; however, any efforts to fully eradicate the pathogen are inadequate and have proven to be unsuccessful. The best efforts against a pathogen lie within maintenance (Steiner, 2000).

The use of chemical applications is one of the most popular as well as successful pathogen and pest control methods. Chemical controls are primarily done in the early spring or fall as a means of preventing infection from occurring or overwintering in the tree till the next season. The most common chemical controls used within the United States fruit tree industry are streptomycin, kasugamycin, oxytetracycline, and copper (Johnson, 2000; Sundin & Wang, 2018). When using an antibiotic for bacterial control, the modes of action vary by the product. The variation impacts how the antibiotic interacts with the host-pathogen, such as by either being bacteriostatic or bactericidal. Bactericidal mode of action is to disrupt the ribosomal complex within the bacterial cell to kill the bacteria and prevent any further reproduction, while bacteriostatic is a growth inhibitor that simply slows down the progression of the bacteria in its conquest attempt to reproduce and spread infection (Johnson, 2000).

Streptomycin is the most commonly used antibiotic in the fruit tree industry for preventing a fire blight infection and has been used for over 50 years (Tancos et al., 2016). Streptomycin was first discovered in 1944, just a few years after the discovery of penicillin in

1928. With the discovery of penicillin and its diverse role in overcoming bacterial infections, antibiotics were soon viewed as having the potential to "cure-all". Streptomycin received its popularity from its diversity of being able to control and kill both gram-negative as well as gram-positive bacteria (Sundin and Wang, 2018). Streptomycin works exceptionally well at controlling fire blight as it is able to reduce the bacterial presence at the initial blossom blight infection stage. By preventing the initial infection from occurring, the likelihood of an outbreak or mass spread of the pathogen is greatly hindered. Streptomycin's mode of action works by inhibiting the growth of *E. amylovora* and prevents it from continuously reproducing, as it is a bactericidal antibiotic. The best time for streptomycin application is during bloom when blossom infection potential is at its highest. The fear of resistance with streptomycin is ever growing. Resistance to streptomycin is most likely to occur when a producer sprays streptomycin more than three times in a season or, when improper recommended applications are used (Breth et al., 2006).

Oxytetracycline, another very popular antibiotic that is used in the fruit tree industry is also known as Tetracycline. Oxytetracycline was discovered later than streptomycin, in the 1950s (Finlay et al., 1950). Similarly, it can work against both gram-negative and positive bacteria; however, it is not bactericidal but instead bacteriostatic. When tested against *P. fluorescens* in three consecutive years (1992, 1993, and 1994), a 10-fold reduction of the bacterial population was observed when compared to a water control (Stockwell et al., 1996).

Kasugamycin is one of the newest antibiotics to be brought into circulation against fire blight. Kasugamycin was first utilized and developed in the 1960s in Japan. Its initial purpose was to be used in rice fields controlling the fungal pathogen that causes rice blast (McGhee & Sundin, 2011; Sundin & Wang, 2018). The use of kasugamycin in the United States and Canada

is primarily for the control of fire blight in fruit tree orchards. Kasugamycin, like streptomycin is bactericidal.

A major conflicts associated with using antibiotics for pathogen control is the potential for resistant bacteria to develop over time. Generally, resistance will occur when the antibiotic is being used too frequently or without proper rotations. In some circumstances, resistant bacteria can arise when the antibiotic is not being applied at its recommended rate, and there is a remaining bacterial community that can become resistant. It is known that antibiotic resistance will arise through the work of horizontal gene transfer (Sundin and Wang, 2018). An alternative to antibiotics is the use of copper. Copper, in some circumstances, can be used as an organic chemical control. However, copper can only be used to control blossom blight in apples when applied prior to an infection occurring (Tancos et al., 2016). Also, copper has the potential to harm the fruit through phototoxicity and russetting (Sundin and Wang, 2018) and can be hazardous to humans by accumulating within the environment (Kering et al., 2019), making it a less than ideal candidate for *E. amylovora* control.

In addition to chemical controls, monitoring weather conditions and prediction models can help a grower know the most likely time fire blight will strike and when to spray. Maryblyt is a strategic, prediction model utilized in the fruit tree industry to predict the optimal time frame to prevent fire blight infection from occurring by applying appropriate bactericides (Biggs et al., 2008). Generally, the combination of temperature and moisture can help researchers and growers predict the potential severity of a fire blight infection, as described previously. Depending on the growing region, prediction models can vary and may be operated by the extension specialist in the area (Johnson, 2000). Furthermore, the use of cultural controls should be used as a first line of defense against fire blight. Cultural controls include pruning damaged tree material 20 to 30

cm from any visible blighted tissue or cankers (Johnson, 2000), removing flowers in young, non-producing trees, prevents the possibility of an outbreak from occurring (Breth et al., 2006), and finally, replacing trees that have an infection that has reached the trunk, this removes the inoculum source from the orchard.

While chemical and cultural controls are the most popular choices for fire blight control, there is an ever-increasing fear of antibiotic resistance. The fear of streptomycin resistance in fire blight has been a concern ever since its discovery in New York in the 1970s (Tancos et al., 2016). Since then, proper rate and application has been stressed to prevent any further resistance from occurring, however, it isn't fool-proof. Biocontrol's can be used as supplemental to existing control methods, such as cultural controls, or can be used as the predominate control method against a bacterial infection. Biological controls predominately consist of fungus and antagonist bacteria. Blossom Protect, a biological control composed of fungi yeast, designed to reduce *E. amylovora* bacterial populations, has been researched in multiple locations of the United States, with varying results. While Blossom Protect may show great potential as a biological control against *E. amylovora*, its fungal composition means a farmer will never be able to spray fungicides in their orchard (Slack et al., 2019). While fungi prove to be of great risk for biological controls, antagonist bacteria research is ongoing in many bacterial research aspects, including *E. amylovora* (Liu et al., 2020; Stockwell et al., 1996; Thomson & Gouk, 2003). Antagonist bacteria work by inhibiting the growth of the infecting bacteria. Generally, by "competing" with the infecting bacteria by producing antibiotics that will reduce the infecting bacterial population. In *E. amylovora* research, the antagonist bacteria, *Pantoea agglomerans* was used as a carrier for bacteriophage, another biocontrol agent, to utilize both the antagonist bacteria, *P. agglomerans* and the bacteriophage to control the population of *E. amylovora*

(Stockwell et al., 1996). Bacteriophage are strain-specific viruses that infect bacterial cells and have been a case of study as an alternative biological control for nearly 100 years (Salmond & Fineran, 2015). The utilization of these naturally occurring organisms has excellent potential to supplement the already existing control methods for fire blight and other diseases.

BACTERIOPHAGE BIOLOGY

For many years now, there has been a growing concern from the research community, the medical community, as well as the general public about repercussions that can stem from our actions—specifically, the concern for antibiotic use and resistance. Antibiotics were first discovered in 1928 by Alexander Fleming in the form of penicillin (Schrader, 2014). This work has led to a global utilization of antibiotics for uses in medicine, animal care, and food production. However, with improper and excessive use, the problem of antibiotic resistance is at the forefront just shy of 100 short years after the discovery of the bacterial-reducing properties of antibiotics. The terms "superbug" and "drug-tolerant" are now widely used in bacterial control research.

Since their discovery, the rate of development of new antibiotics is occurring at a slower rate than resistance is evolving to current antibiotics; therefore, researchers have been stimulated to look in other directions for control materials for bacterial diseases. Curiosity has led many to search for and study biological controls. Biological control can be defined as the utilization of one pest or pathogen in an efforts to control another. This is referred to as Integrated Pest Management (Biddinger, 2017), a strategy for reducing and preventing pests and pathogens through habitat manipulation, resistant varieties, and cultural practices (Flint, 2012). Ideally, biological controls work by either removing the threat/ pest completely or altering the

environment conducive for the pest and or pathogen that is the targeted issue. Examples of biological controls that are used in the tree fruit industry are the use of lady beetles to control aphids, antagonistic bacteria that can inhibit bacterial pathogens through either antibiosis or competition, and strain-specific bacteriophage to control bacterial pathogen populations on plant surfaces (Biddinger, 2017).

Bacteriophage were first discovered in 1915 and rediscovered in 1917 by the microbiologist Felix d'Herelle, who would go on to give bacteriophage their name, which translates to bacteria-eater (D'Herelle, 1917). D'Herelle generated this name after observing that phage would cause reductions in total bacterial populations. In the 1940s, phage were visualized for the first time by using electron microscopy; however, by this time, antibiotics had already been discovered and were being utilized in such mass quantity that phage therapy could not compete. In addition, phage research was questioned as truly reliable and effective by the Council of Pharmacy and Chemistry of the American Medical Association in the 1930s (Salmond & Fineran, 2015; Summers, 2001). While phage were not specifically being studied for their ability to control bacterial pathogens, the biological properties of the phage-bacteria interaction were being discovered. These properties included phage lysis of bacterial cells, the evolution of resistance to phage infection in bacteria, and the ability of phage to overcome that resistance to once again lyse the bacterial cells (Kering et al., 2019). Unlike antibiotics, phage have the ability to overcome bacterial resistance when it occurs. This phenomenon has been studied and observed since the mid 20th century to further understand genetics (Kortright et al., 2019). However, because of the rise of antibiotics and also the testing and utilization of antibiotics in some plant agriculture applications, including for fire blight, phage as a biocontrol has been put "on the back burner".

The magic of what makes bacteriophage a good biological control lies within how the bacteriophage reproduce. The phage reproductive cycle or life cycle can be broken down into two pathways—the lytic cycle and the lysogenic cycle (Salmond and Fineran, 2015). Lytic phage are desired in the biocontrol, and bacterial control industry, as the main outcome is the lysing of bacterial cells and the production of more phage. Initially, when a bacteriophage identifies its strain-specific bacterial host, it will utilize binding proteins to attach to the bacterial cell. Once attached, the phage will inject its viral DNA, also known as a prophage, into the bacterial cell. The viral genome will then begin to be replicated by the bacterial replication machinery; this process is followed by transcription and translation of the phage genome and assembly of new bacteriophage. Once an abundance of phage has built up within the bacterial host cell, a phage protein is produced that lyses the host cell, releasing the population of phage into the environment where they can attach to new bacterial cells and repeat the process (Rostøl & Marraffini, 2019; Salmond & Fineran, 2015).

The lysogenic cycle of bacteriophage differs from the lytic cycle in that host bacterial cells are not killed. Instead, once the prophage has been injected into the bacterial host, it will become integrated into the bacterial DNA. This bacterial cell can still replicate and function; however, it now has additional DNA. Lysogenic cells do have the ability to become lytic. This occurs when the prophage is stimulated to excise from the bacteria DNA and begin the lytic cycle (Rostøl and Marraffini, 2019). This process is called induction and is typically induced by agents that cause DNA damage to the host bacterial cell (Steward, 2018)

Bacterial cells have evolved a variety of mechanisms to resist or inhibit phage infection. As described previously, the phage lysis cycle begins with a bacteriophage attaching to a bacterial cell by the utilization of binding proteins. There are many theories that question

whether the existence of bacterial cells within a biofilm provides protection against phage attachment. It has been shown that bacterial cells located at the exterior of a biofilm may not be as safe from phage attachment as those towards the interior of the biofilm. In addition, *E. coli* cells in early stages of biofilm development were more sensitive to infection by T7 bacteriophage (Rostøl and Marraffini 2019, Vidakovic et al., 2018)

Erwinia amylovora produces three exopolysaccharides, amylovoran, cellulose, and levan, which play major roles in virulence. The amount of EPS a pathogen produces also determines bacteriophage infectiveness. It was proven through the use of *invitro* phage lysis assays that bacteriophage that belong to the Myoviridae family prefer LEPS (low EPS producing bacteria) strains of *E. amylovora* as they produced clear plaques, with little EPS, while phage of the Podoviridae family is more virulent against HEP (high EPS producing bacteria) *E. amylovora* strains (Roach et al., 2013). In addition to phage having specificity to EPS levels, some also have the extraordinary ability to break down EPS through the production of a depolymerase enzyme. This process is only carried by the Podoviridae bacteriophage and can make the phage more advantageous against bacteria. With the ability to break down EPS, there is greater accessibility to the cell surface where the phage can carry out the lytic infection process (W. S. Kim & Geider, 2000)

USE OF BACTERIOPHAGE FOR PLANT DISEASE MANAGEMENT

It has been estimated that the total global population of bacteriophage is 10X that of the total global population of bacteria (Jończyk et al., 2011) which makes phage the most abundant organism in the world. Also, it is highly likely that every bacterial cell on earth is susceptible to at least one bacteriophage. However, because most phage are strain-specific, utilizing them for

biological control can be very challenging and highly dependent on the diversity of the target bacterial pathogen. Thus, phage biological controls cannot be based on a single phage species, as the single phage type would likely not lyse all pathogen strains, and the chances of the pathogen evolving resistance would be significantly greater. Therefore, for disease control applications, different phage are typically combined in what is known as a "cocktail ". Phage cocktails can be described as a combination of like phage that infects the same or similar species of bacteria. Combining several phages into one cocktail increases the chances of targeting the entire bacterial pathogen population and reduces the impact of resistance evolution toward any one phage in the mix. Having the ability to prevent resistance will allow for greater longevity of the product and a more successful reduction of bacterial population size. To create a phage cocktail, it is extremely important to have knowledge of the bacterial host range of the utilized phage. The host range can be studied in several ways; for example, a recent analysis by Gayder et al (2019) used QPCR to test 10 bacteriophage against 106 different *E. amylovora* strains. This work resulted in a quantitative understanding of infection by individual phage against a diversity of *E. amylovora* (Gayder et al., 2019). This knowledge is a huge step in the right direction to developing effective and potent phage cocktails. The knowledge of how virulent a single phage is versus another allows researchers to strategically choose the most effective and aggressive phages to include in their cocktail (Liu et al., 2020).

While phage are only specific to bacteria, their diversity is anything but lacking. Phage can be found wherever bacteria are, from soils to the oceans, sewers, in human and animal guts to the meat and produce humans and animals consume making phage abundant and present wherever scientists look. In the animal agriculture production sector, phage are being used in substitute for antibiotics. The animal agriculture industry is one of the greatest consumer of

antibiotics, being used for the control and preventative spread of diseases and pathogens. This will, in turn, create a healthier animal and will allow the animal to gain weight quicker (Svircev et al., 2018). By the year 2030, it is expected that the animal agriculture industry will have increased its antibiotic usage by 67% of what is used in the industry today (Gelband, H. et al., 2015). This leads to the question, if the animal industry will be increasing at such a dramatic rate? What can we expect from the fruit tree industry? By introducing bacteriophage as bacterial control agents it is probable that the need for antibiotics won't have to sky-rocket. To demonstrate, phage have been added into the feed of hogs to reduce intestinal bacterial pathogens that hinder the hog from gaining weight at a quicker pace (K. H. Kim et al., 2014). While in the aquaculture industry, phage are increasing the survival rate of salmon and zebrafish larvae against the bacterium, *Vibrio anguillarum* (Higuera et al., 2013; Silva et al., 2014) proving that phage, in this instance, possess a significant role as an antibiotic substitute.

Bacteriophage are also gaining a lot of attention in the plant sector of agriculture. The plant production industry is constantly at battle against pathogens and pests that cause infection and are significantly affected by antibiotic resistance, while the public demands more natural control methods that don't contain synthetic chemicals. One industry in particular, the grape industry, has recently been battling the pathogen, *Xylella fastidiosa*. *X. fastidiosa*, is more commonly known to grape producers as, Pierce's disease. This disease is comparably more challenging than others as it infects the xylem of the grape plant. Currently, the main form of control is applying neonicotinoid pesticides. These pesticides have shown to take a toll on the winterization of bees and other pollinators in areas it is applied, making many producers weary to use it (Buttimer et al., 2017; Das et al., 2015). Research has concluded that the addition of a phage cocktail was able to prevent pathogen symptoms from arising and keep the bacterial

population controlled (Das et al., 2015). In addition to grapes, the fruit tree industry has been working with bacteriophage for many years, especially in the potential efficacy of controlling fire blight in apples and pears (Table 1).

Table 1: Diversity of bacteriophage strains used, classified, or researched for biological control properties.

PATHOGEN	PHAGE	SOURCE	ASSAYS CONDUCTED	DATE
<i>E. amylovora</i>	φ9-5	(Lehman 2005). Ch 5	field	2005
<i>E. amylovora</i>	φ21-4			
<i>E. amylovora</i>	φ35-5			
<i>E. amylovora</i>	φ46-1A2			
<i>E. amylovora</i>	φvB_EamM_Y3	(Buttimer et al., 2018)	classification	2018
<i>E. amylovora</i>	φEa2345-6	(Boulé et al., 2011)*	Greenhouse/ in vitro	2011
<i>E. amylovora</i>	φEa2345-19			
<i>E. amylovora</i>	φEa21-4			
<i>E. amylovora</i>	φEa1598-6			
<i>E. amylovora</i>	φEa1594-24			
<i>E. amylovora</i>	φEa1594-26			
<i>E. amylovora</i>	φEa1598-19			
<i>E. amylovora</i>	φEa1337 -26			
<i>E. amylovora</i>	φS1	(Erskine, 1973)	In vitro	1973
<i>E. amylovora</i>	φH4A	(Schwarczinger et al., 2011)*	In vitro, flowers in tubes	2011
<i>E. amylovora</i>	φH5A			
<i>E. amylovora</i>	φH6			
<i>E. amylovora</i>	φH11			
<i>E. amylovora</i>	φY2	(Born et al., 2017) (Born et al., 2015)*	In vitro	2015,2017
<i>E. amylovora</i>	φEa1h	(Müller et al., 2011)	In vitro	2011
<i>E. amylovora</i>	φEa100	(Ritchie, 1979)	In vitro/ classification	1979
<i>E. amylovora</i>	φEaJ08T			
<i>E. amylovora</i>	φEaK08T			
<i>E. amylovora</i>	φEa104			
<i>E. amylovora</i>	φEa116			
<i>E. amylovora</i>	φEaJ08C			
<i>E. amylovora</i>	φPEa1(h)			
<i>E. amylovora</i>	φPEa1(nh)			
<i>E. amylovora</i>	φPEa2(nh)			

Table 1 (cont'd)

<i>E. amylovora</i>	φPEa2(h)			
<i>E. amylovora</i>	φPEa3(n)			
<i>E. amylovora</i>	φPEa4(h)			
<i>E. amylovora</i>	φPEa5(h)			
<i>E. amylovora</i>	φPEa6(h)			
<i>E. amylovora</i>	φPEa7			
<i>E. amylovora</i>	φPEa8(h)			
<i>E. amylovora</i>	φPEa 12(h)			
<i>E. amylovora</i>	φPEa 13(h)			
<i>E. amylovora</i>	φPEa15			
<i>E. amylovora</i>	φPEa 16(h)			
<i>E. amylovora</i>	φEa1	(Elise L Schnabel & Jones, 2001)	In vitro	2001
<i>E. amylovora</i>	φEa7			
<i>E. amylovora</i>	φEa100			
<i>E. amylovora</i>	φEa125			
<i>E. amylovora</i>	φEa116C			
<i>E. amylovora</i>	φphiEaP-8	(Park et al., 2018)	In vitro/isolation	2018

*-Papers that did tests/research on these phage, not just classified/altered them.

The isolation and identification of an *E. amylovora* specific phage is the first step in utilizing phage for the control of fire blight. Secondly, identifying the proper time and application method for the phage to lyse at an optimal rate is key (Jones et al., 2013). The phage application process has been analyzed to determine the best possible application technique and time that will allow the greatest virulence from the phage. When applied periodically to the tree without any indication of *E. amylovora* being present, the control is very low. However, when the phage are applied with the bacteria, or during peak bloom when infection is most likely to occur, there is a significantly greater chance of the phage lysing the bacteria and preventing an infection (Schnabel et al., 1999). As an alternative application method, in 2005, Canadian

researcher, Susan Lehman, studied and developed a methodology of utilizing bacteriophage as a biological control in the apple industry. Lehman tested treatments of bacteriophage combined with *Pantoea agglomerans*, an antagonist bacteria, described as a "carrier" bacteria to promote the movement of the bacteriophage. This methodology was selected as *P. agglomerans* would move similarly to *E. amylovora* and would carry the bacteriophage straight to the *E. amylovora* bacteria for lysing. Statistically, these results did not differ in their control than that of a streptomycin antibiotic control application (Lehman, 2007). The proper application timing of bacteriophage also plays a large role in how successful the phage may be. Sunlight irradiation is considered to one of the greatest reasons for reducing a phages virulence when applied in nature (Iriarte et al., 2007). The amount of light exposure and UV present has a direct correlation with how quickly bacteriophage will degrade. Meaning, the best times to apply bacteriophage to fruit trees for fire blight prevention is during the late afternoon or evening, when the sun is setting and UV rays are not as intense (Iriarte et al., 2007; Kering et al., 2019).

Bacteriophage are strain-specific, naturally occurring bacterial-infecting viruses. Current tree fruit production systems rely heavily on the use of antibiotics and copper to reduce populations of the fire blight pathogen *E. amylovora*. Recently, resistance to the antibiotic streptomycin has been increasing in *E. amylovora* populations, and there is also concern from consumers as to what is being applied onto their foods. This has led to an overall increased interest in alternate pathogen controls that are known to be more natural. The category of biocontrol for fire blight is very broad, with varying results depending on the specific climate and location. Bacteriophage have proven to have significant potential to be a beneficial supplementary factor in controlling fire blight and reducing populations of the bacterial pathogen *E. amylovora* on flowers. My research builds on this body of knowledge as I am actively

utilizing *E. amylovora* specific bacteriophage in a controlled field and lab setting. The purpose of my research is to better utilize bacteriophage as a biological control. This is done by understanding the bacteriophage's weaknesses, such as sensitivity to ultraviolet (UV) lighting radiation. UV light radiation has been noted as reducing the cell-lysing causing cell degradation and deteriorating the ability of bacteriophage, preventing the phage from properly lysing (Iriarte et al., 2007; Kering et al., 2019). In my research, I have explored alternative methods to preserve the bacteriophage from degradation through the addition of materials to the phage application. Such materials include carrot juice, peptone, and Surround (kaolinite clay). These materials were hypothesized to be efficient in protecting the bacteriophage from UV ray radiation degradation due to the nature of their consistency, and color. Having the ability to protect bacteriophage from UV degradation could significantly increase bacteriophages capabilities for pathogen control in a real-world, field setting.

CHAPTER TWO:

ANALYZING THE RELATIONSHIP BETWEEN UVR IRRADIATION AND ERWINIA AMYLOVORA

INTRODUCTION

Erwinia amylovora, a devastating bacterial pathogen that is known to wreak havoc on many orchards across the world every year, is economically a very important pathogen to control. In 2016, the apple industry in New York directly contributed 1.3 billion dollars to the economy (Schmit et al., 2018). Making the apple industry responsible for thousands of jobs, and the livelihood of many. The control of *E. amylovora* is heavily reliant on chemical controls, specifically, antibiotics such as streptomycin, kasugamycin, and oxytetracycline. Copper is also a commonly used chemical in *E. amylovora* control (Johnson, 2000; Sundin & Wang, 2018). However, with the increasing concern of antibiotic resistance by both consumers and researcher, the desire for alternate control methods is ever increasing. In the late 1990s, a research group from Oregon State University, examined the effects of utilizing antagonist bacteria along with antibiotics for the control of fire blight. Overall, the disease incidence was only ever controlled at a maximum of 50% (Stockwell et al., 1996). While combining antagonist bacteria with antibiotics may show some control, antibiotics alone typically provided greater, more reliable control results. More recently, additional biological controls, such as *Bacillus*-based materials, yeasts, and bacteriophage have exhibited significant-greater potential to protect against disease incidence compared to antagonistic bacteria.

Bacteriophage have been widely studied for plant disease control, and especially for control potential of fire blight (Jones et al., 2012; Schnabel et al., 1999). Many studies of individual phage have been conducted by the Svircev lab at Agriculture and AgriFood Canada;

Vineland, Ontario. An extensive host range study using 106 *E. amylovora* strains indicated that two phage, ϕ 31-3 and ϕ 21-4, were good biocontrol candidates (Gayder et al., 2019). The deployment of bacteriophage in biological control is also dependent on phage survival in the environment. It is known that many phage are susceptible to ultraviolet radiation (UVR) damage (Born et al., 2015; Jones et al., 2012). Solar UVR is composed of UVA and UVB wavelengths. A total of 95% of the atmospheric UVR is in the UVA wavelength range (Jacobs et al., 2005), of 320 to 400 nm. The remaining 5% of UVR that penetrates through the atmosphere is UVB. UVB radiation, 290 to 320 nm wavelengths, is known to cause direct DNA damage to bacterial cells. These wavelengths produce pyrimidine dimers in the DNA that, if unrepaired, can quickly cause cell death. UVC radiation, having the shortest wavelengths, < 290 nm, and is the most intense UV that creates the most cell damage out of the three, but does not penetrate the atmosphere (Jacobs et al., 2005). However, when exposing a bacterial cell to UVC in a controlled lab setting, low doses can cause reductions in the active bacterial population, due to the cellular damaging properties.

The amount of UVR degradation of bacteriophage varies by the type and concentration of protectants, and the type of UVR the bacteriophage is exposed to. To protect the bacteriophage from UVR degradation several types of protectants can be utilized. Specifically, protectants are chosen based off of properties that may benefit the phage. Properties such as a high pigmentation, viscous consistency, or the protectants ability to bind to bacteriophage. There are endless possibilities of potential protectants that may fit that criteria. In studies of developing protectants beneficial to phage, skim milk has been utilized to assist the bacteriophage in adhering to a leaf surface while also keeping the phage from being degraded by UVR (Balogh et al., 2003; Balogh et al., 2008; Iriarte et al., 2007). Other foods such as carrot juice, red pepper

juice, and beet root, are known for absorbing UV-rays, thanks to their phenolic compounds, and have been used in bacteriophage protective studies (Born et al., 2015). Other ingredients also included clays (kaolinite and montmorillonite), casein, and peptone (Born et al., 2015; Vettori et al., 2000).

The primary goals of these experiments were to identify the best possible UV-protectant while also minimizing the amount of protectant needed to ensure necessary coverage and protection from UVR. In an applied field setting, the addition of another spray application can be costly. Being able to minimize the amount of product applied while still optimizing UVR protection to the phage was a main priority in this research. Economically, it is a goal of producers to obtain the highest level of product coverage while obtaining optimal protection with the least amount of money invested.

MATERIALS AND METHODS

For these *in vitro* experiments the bacteriophage were combined with potential UV-protectants, peptone, Surround (Kaolinite clay), and carrot Juice. Both peptone and carrot juice were used previously as UV-protectants in a study conducted by Born et al (Born et al., 2015). This study was done predominately *in vitro* using 96 well plates and soft agar overlays to determine the remaining level of PFU after UVR exposure. These results concluded that high rates, such as 50 mg/ml of peptone and 10% carrot juice, demonstrated the ability to protect the bacteriophage by upwards of two logs than if UV-protectants had not been added. The addition of Surround to this project was inspired by kaolinites viscous consistency, and cloudy pigmentation. This comes with knowing that bacteriophage have been proven to bind to clay particles in a natural environment (Vettori et al., 2000). We hypothesized that the bacteriophage

would bind to the viscous, pigmented clay, and would be protected from UV wavelengths that causes DNA damage. Surround is currently approved by the Organic Material Review Institute (OMRI) and is regulated to be used as an organic pest control on vegetables and fruit trees. For a group in Italy, they studied the interaction of PBS1 bacteriophage, specific to *Bacillus subtilis*, with two clay minerals, montmorillonite, and kaolinite. The mixture of phage and clay was irradiated with UVC radiation (254 nm) for a maximum time of 30 minutes. Samples were taken at 0, 1, 2, 5, 10, and 30 minutes. Titters were calculated to accurately determine a level of inactivation among the bacteriophage. It was concluded by the group that without clay binding the bacteriophage decreased significantly within the first 5 minutes while bacteriophage with clay association held constant in titer for up to 10 minutes of irradiation. A 30 minute irradiation was essential to compare a decrease in bacteriophage between the free phage and phage with clay (Vettori et al., 2000).

Bacteriophage (1.6×10^8 plaque-forming units [PFU]) were mixed in a petri dish (60mm x 15mm) with phage buffer (Tris HCl- 10 mM, MgSO₄ -10 mM, NaCl- 150 mM) for a total volume of 5 ml. The recommended rate of application for Surround is 0.454 kilograms of Surround per 7.57 liters of water. In this experiment, the UV-protectants were diluted to 8%, 4%, 2%, and 1% of the recommended application rate. The peptone, which does not have a regulated, recommended application rate, was tested at 50 mg ml⁻¹, 10 mg ml⁻¹, and 5 mg ml⁻¹, and the carrot juice was tested at 1% and 10% concentrations. Each treatment was tested against both Φ31-3 and Φ21-4 in three separate experiments, with three replications per experiment. In addition to the bacteriophage strains, in the UVA experiment an additional bacteria, *P. chloraphis* 06, was used as a control for comparison. Three replications were conducted for each treatment, and each treatment was repeated three times. A UV lamp was turned on 15 minutes

prior to intended use, either UVC, UVB, or UVA. The petri dishes were placed under the lamps, allowing UVR exposure from 250 J m^{-2} to 2000 J m^{-2} for UVB, 666,000 to 2,000,000 J m^{-2} for UVA, and 90 to 360 J m^{-2} for UVC. Samples (100 μl) were taken from the petri dishes after the appropriate UVR exposure. In between each sampling time point, the petri dishes were thoroughly swirled to make sure the phage and buffer mixture were well mixed to prevent self-shading. The UVA phage solutions were placed on a constant shaker table set at a low speed as to not spill the solution. After irradiation, appropriate serial dilutions were done to later determine the amount of active PFU through a soft agar overlay technique, of pouring molten agar containing the phage and *E. amylovora* over an existing agar plate (Rabiey et al., 2020). For this technique, along with the serially diluted bacteriophage, *E. amylovora* Ea110 bacteria was concentrated to 0.2 OD_{600 nm}, using the absorbance on the Tecan Spark plate reader. The overlay was poured on to Kings B agar plates and placed in a 28 C incubator overnight. After 24 hours plaques were counted.

Phage plaque counts were transformed by $\log_{10}$ after calculation from counting plates. Percent phage survivability over the time course of the experiments was calculated by the equation $(n_2 - n_1) / n_1$. Since treatments negatively impacted growth rates, values for subsequent analysis were inverted to calculate an area under the curve (AUC) for each transformed replicate experiment using R package “growth curve” (Sprouffske and Wagner, 2016). To further investigate differences between treatments, analysis of variance (ANOVA) was performed on the calculated AUC values and treatments were separated via Tukey’s HSD (Slack et al., 2021). All statistical computing was performed in R: A language and environment for statistical computing (Team, R.C, 2013).

RESULTS

EFFECT OF UVR IRRADIATION ON Φ 21-4, AND Φ 31-3 WITHOUT UVR PROTECTANTS AND WITH PEPTONE, SURROUND, AND CARROT JUICE

UVC-UVC is the most aggressive UVR as it deactivates phage the quickest out of all three radiations. When exposing ϕ 21-4 and ϕ 31-3 to UVC without any protectants there is a very steady decrease in PFU's (plaque forming units). Over the course of 240 seconds of exposure both phage experience a reduction in viable phage and have 35% of the phage remaining active for both the bacteriophage. (Fig. 1 and 2). To prevent the rapid loss of bacteriophage, potential UV shielding compounds, Peptone, Surround (kaolinite clay) and carrot juice were added to the bacteriophage mixtures. The additive of peptone was tested at 50 mg ml⁻¹, 10 mg ml⁻¹, and 5 mg ml⁻¹ (Fig. 3 and 4). Results showed significant protection by all peptone doses in comparison to the experiments without any UV- protectants. Peptone at 50 mg ml⁻¹ showed the best protection with less than 5% loss of PFU, the 10 mg ml⁻¹ had a slight, but minimal decrease in PFU across the 360 jm²⁻¹, with 90% of the phage remaining active and 5 mg ml⁻¹ which demonstrated a deactivation of 20% of the phage, keeping the remaining 80% active. This is a significant increase from the 65% loss when tested without protection. The Surround was added to the bacteriophage as dilutions of 8%, 4%, 2%, and 1% the recommended label rate (Fig. 5 and 6). For both ϕ 21-4 and ϕ 31-3, Surround at 8% protected the phage thoroughly with the greatest loss of PFU being 5%, the 4% kept a survivability of 90%, while the 2% and 1% demonstrated a protection of roughly 50%. Carrot juice was the last protectant tested with ϕ 21-4 and ϕ 31-3 under UVC (Fig. 7 and 8). The carrot juice, concentrated at 1% and 10%, for both bacteriophage, only showed protection at a maximum of 50%.

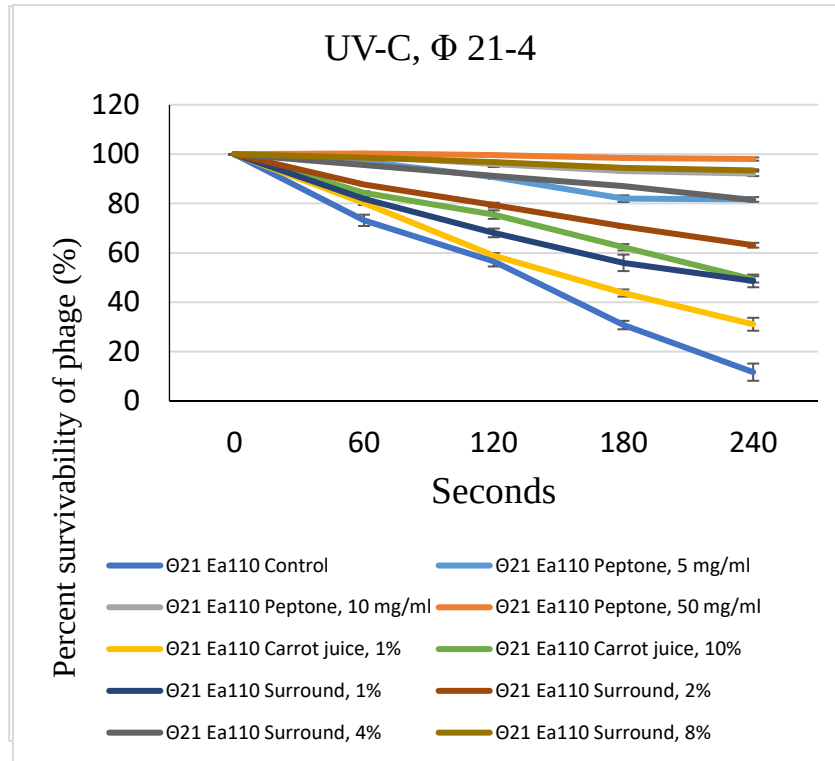


Figure 1: Percent survivability of Φ 21-4 exposed to UVC with all treatments

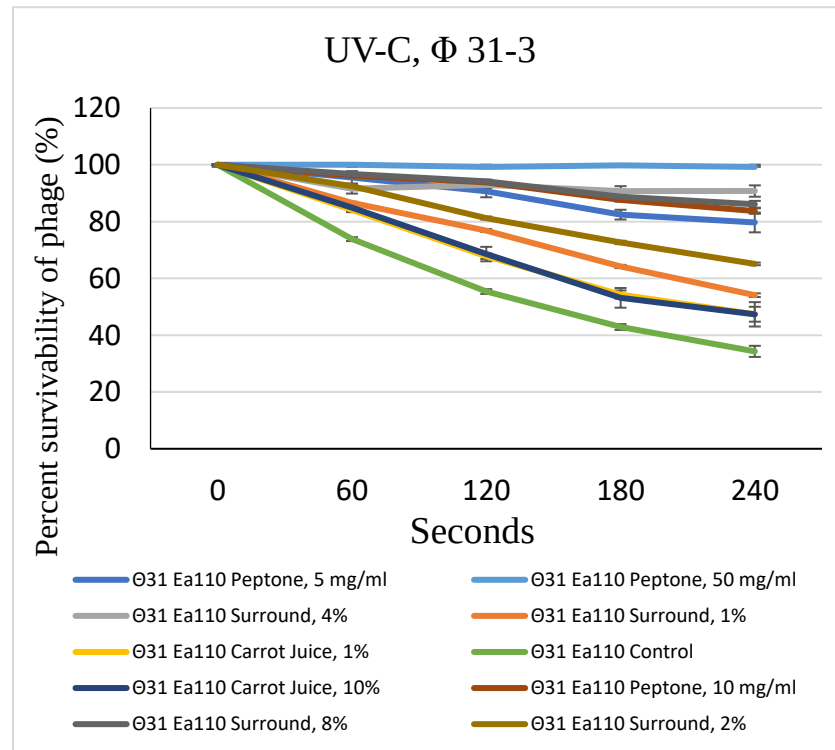


Figure 2: Percent survivability of Φ 31-3 exposed to UVC with all treatments

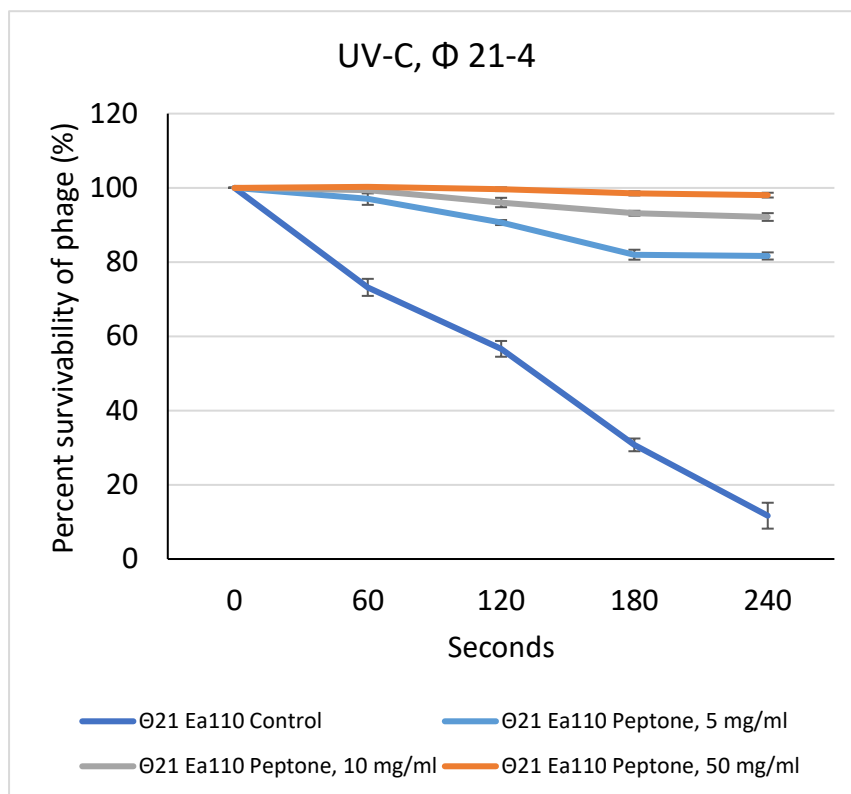


Figure 3: Percent survivability of Φ 21-4 exposed to UVC irradiation with 50, 10, and 5mg ml⁻¹

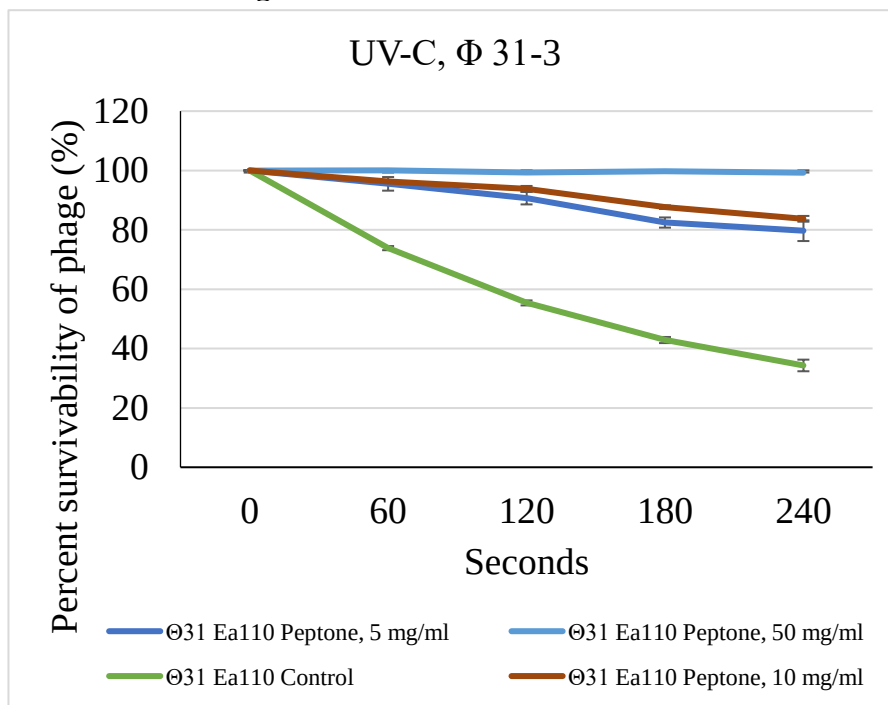


Figure 4: Percent survivability of Φ 31-3 exposed to UVC irradiation with 50, 10, and 5mg ml⁻¹

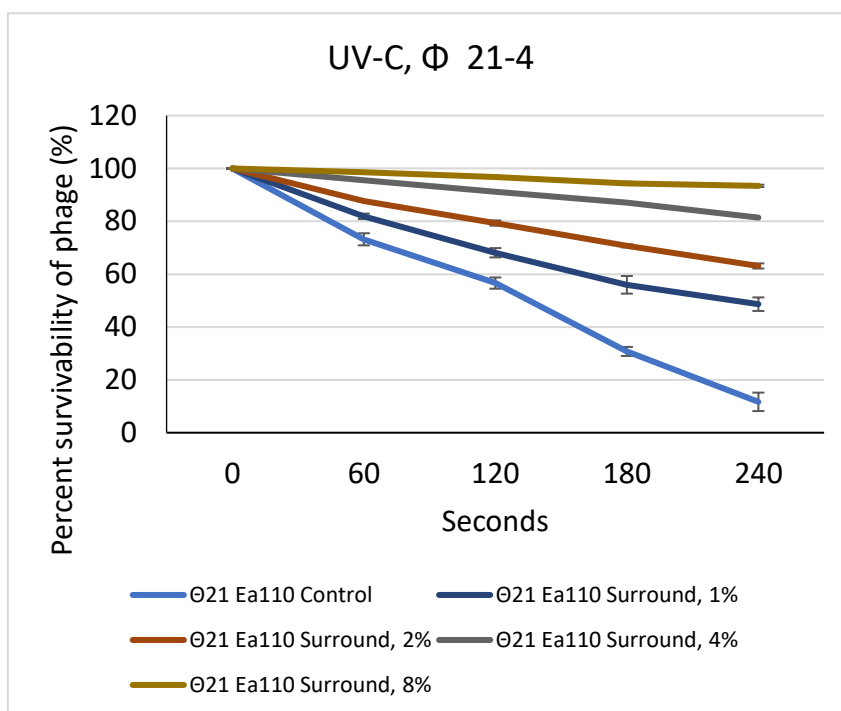


Figure 5: Percent survivability of Φ 21-4 exposed to UVC irradiation with dilutions of 8%, 4%, 2%, and 1% the recommended rate of Surround

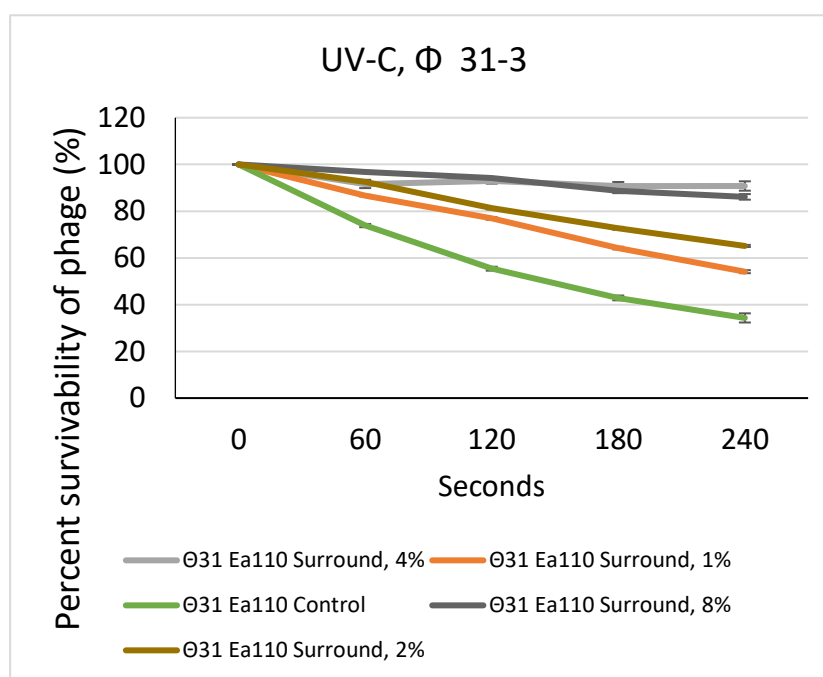


Figure 6: Percent survivability of Φ 31-3 exposed to UVC irradiation with dilutions of 8%, 4%, 2%, and 1% the recommended rate of Surround

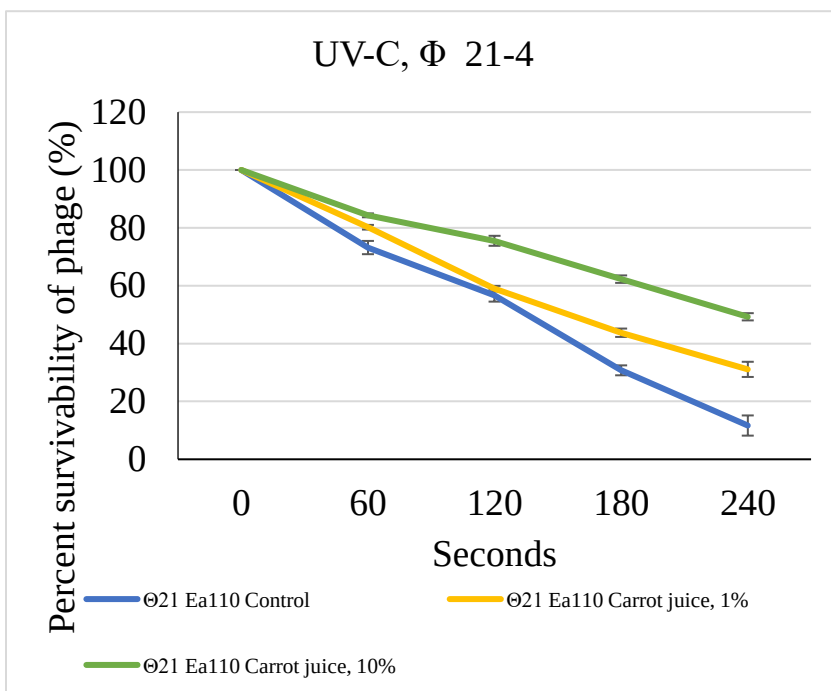


Figure 7: Percent survivability of Φ21-4 exposed to UVC irradiation with 1% carrot juice and 10% carrot Juice

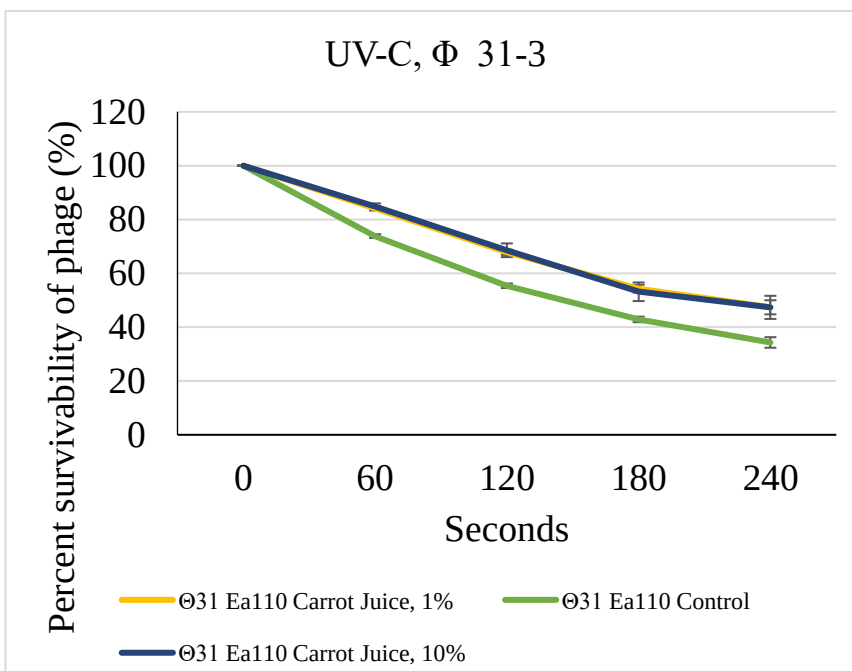


Figure 8: Percent survivability of Φ31-3 exposed to UVC irradiation with 1% carrot juice and 10% carrot Juice

Table 2: AUC, Standard deviation, and HSD >0.05 for UV-C experiments

	Φ 21-4, UVC		Φ 31-3, UVC	
Treatment	Average Area under the Curve (AUC) and Standard Deviation	HSD >0.05	Average Area under the Curve (AUC) and Standard Deviation	HSD >0.05
Ea110 Control	114.1 ± 4.3	A	97.3 ± 2.3	A
Ea110 Carrot juice, 1%	91.5 ± 4.9	AB	72.4 ± 12.3	B
Ea110 Carrot juice, 10%	62.6 ± 6.7	CD	73.1 ± 17.3	AB
Ea110 Peptone, 5 mg/ml	25.5 ± 4.2	EF	24.0 ± 5.5	D
Ea110 Peptone, 10 mg/ml	10.0 ± 0.8	F	18.3 ± 3.8	D
Ea110 Peptone, 50 mg/ml	2.3 ± 0.6	G	1.7 ± 0.14	E
Ea110 Surround, 1%	72.2 ± 13.6	BC	57.0 ± 3.4	B
Ea110 Surround, 2%	47.5 ± 5.3	DE	42.6 ± 2.3	C
Ea110 Surround, 4%	21.1 ± 3.5	E	15.1 ± 2.31	D
Ea110 Surround, 8%	8.6 ± 2.0	F	14.9 ± 3.2	D

UVB- For the UVB exposure experiments the “Naked” bacteriophage, without any additional UV-protectants, decreased in available, active phage over the course of the exposure. Both the $\Phi 21-4$ and $\Phi 31-3$ PFU’s were reduced by a total of 25% with exposure to 2000 Jm^{-2} , as demonstrated in (Fig.9 and 10). While the addition of UV- protectant peptone, at 10 mg ml^{-1} and 5 mg ml^{-1} , comparably, keeps both the $\Phi 21-4$ and $\Phi 31-3$ from reducing its viability by 10 to 15%. (Fig.13 and 14). The amount of PFU remaining after 2000 Jm^{-2} of exposure was significantly greater than when the two bacteriophage, $\Phi 21-4$, and $\Phi 31-3$, were exposed without UV-protectants. With the addition of Surround similar protective qualities were recorded (Fig. 11 and 12). The 8% Surround showed the greatest amount of protection both $\Phi 21-4$ and $\Phi 31-3$, the phage remained at 95% survivability. Comparably, the 4% concentration maintains a similarly constant survival of 90 to 95% . Finally, the 1% and 2% concentrations for both $\Phi 21-4$ and $\Phi 31-3$ showed nearly identical loss of PFU, reducing the amount of active phage to only 80%, the greatest amount of PFU lost in the Surround trials. However, the Surround trials with loss of 20% (1% and 2%), still held greater protection from UV inactivation than if no UV-protectant were added.

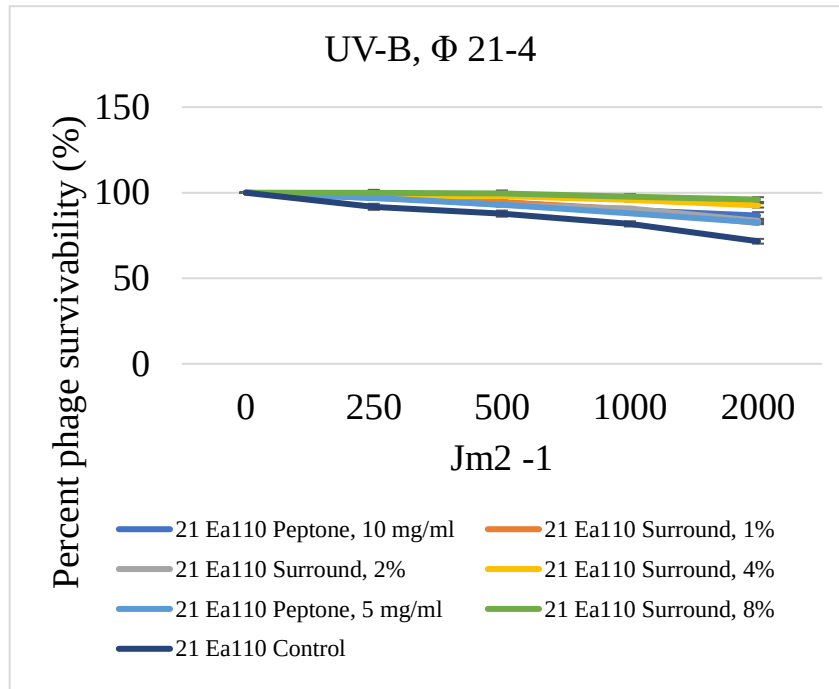


Figure 9: Percent survivability of Φ 21-4 being exposed to UVB irradiation with all treatments

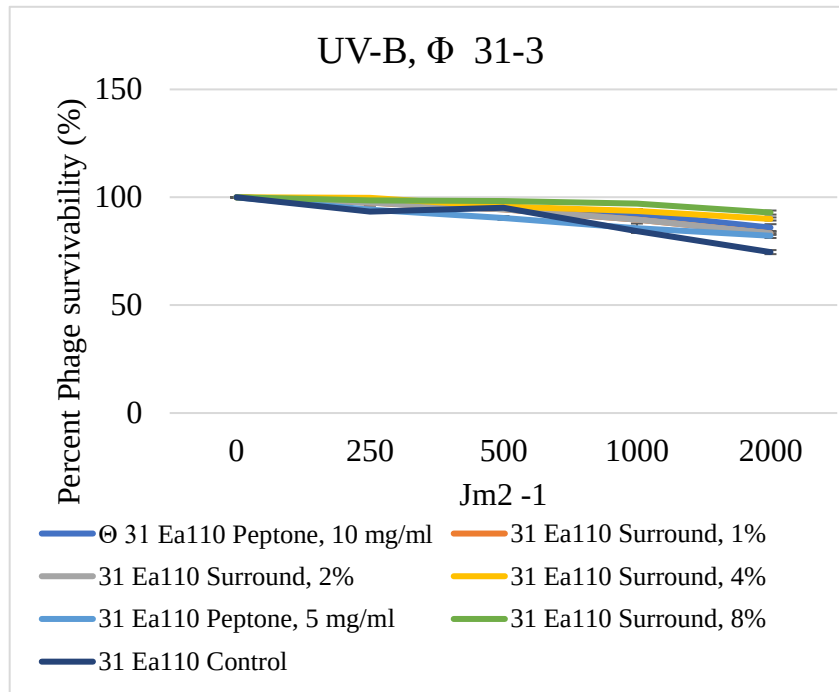


Figure 10: Percent survivability of Φ 31-3 being exposed to UVB irradiation with all treatments

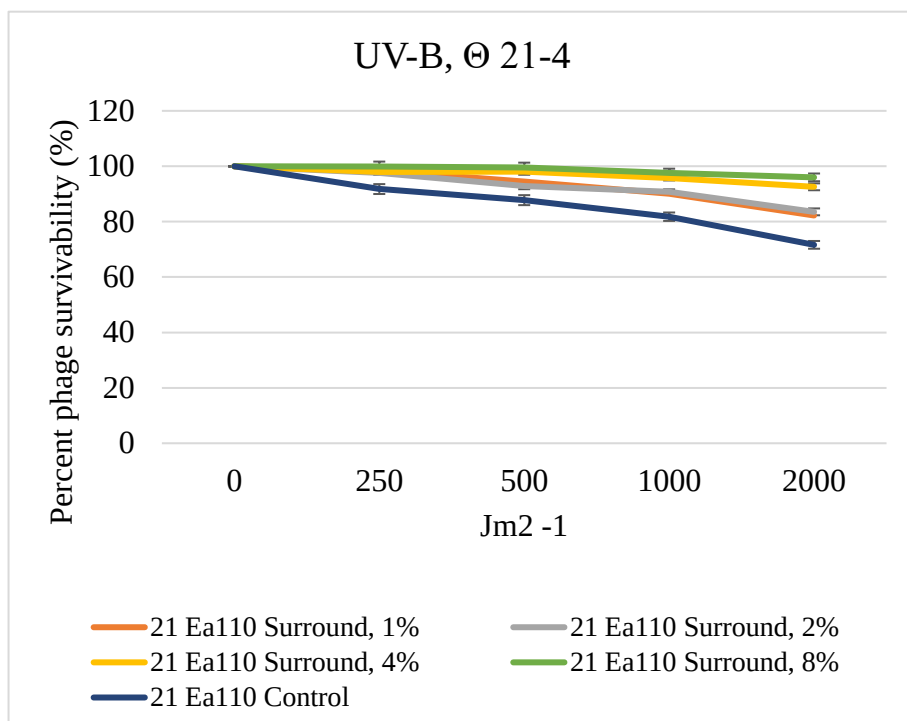


Figure 11: Percent survivability of Φ 21-4 exposed to UVB irradiation with dilutions of 8%, 4%, 2%, and 1% the recommended rate of Surround

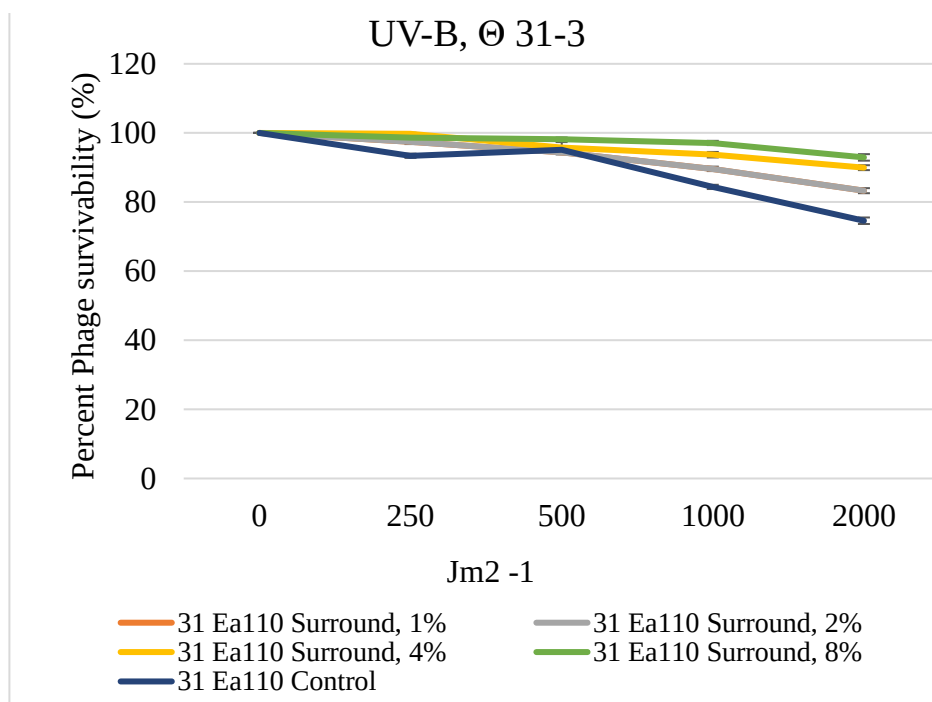


Figure 12: Percent survivability of Φ 31-3 exposed to UVB irradiation with dilutions of 8%, 4%, 2%, and 1% the recommended rate of Surround

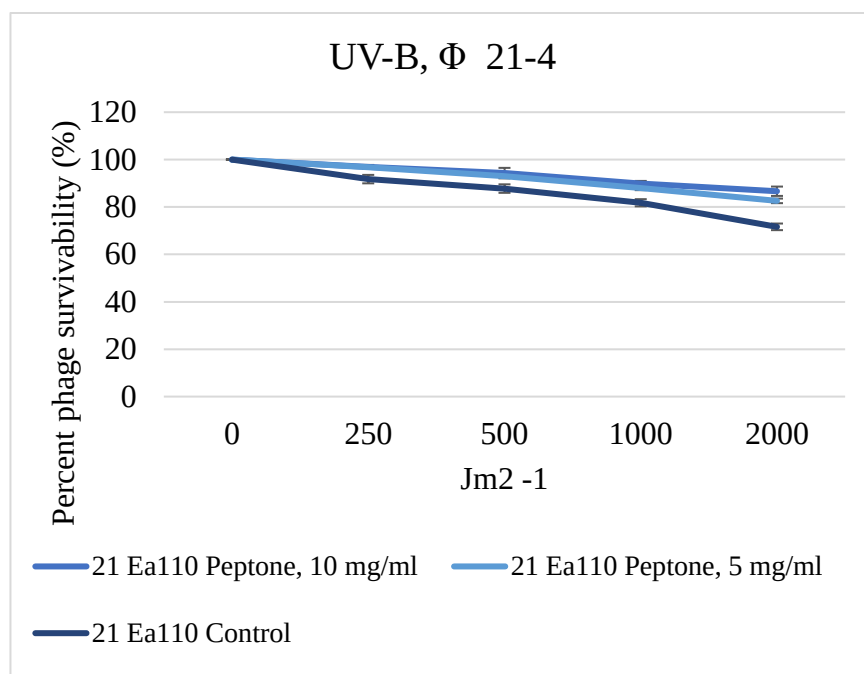


Figure 13: Percent survivability of Φ 21-4 exposed to UVB with 5, and 10 mg ml⁻¹ Peptone

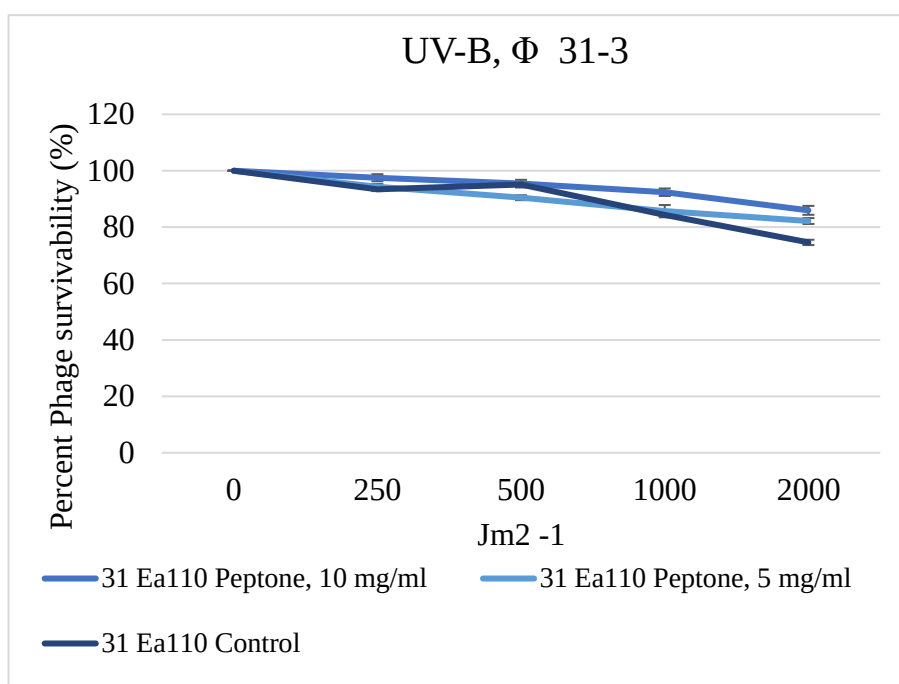


Figure 14: Percent survivability of Φ 31-3 exposed to UVB with 5, and 10 mg ml⁻¹ Peptone

Table 3: AUC, Standard deviation, and HSD >0.05 for UV-B experiments

	Ø21, UVB		Ø31, UVB	
Treatment	Average Area Under the Curve (AUC) and Standard Deviation	HSD >0.05	Average Area under the Curve (AUC) and Standard Deviation	HSD >0.05
Ea110 Control	407.4 ± 69.1	A	290.3 ± 100.8	A
Ea110 Peptone, 5 mg/ml	211.5 ± 12.7	B	246.7 ± 60.5	A
Ea110 Peptone, 10 mg/ml	171.9 ± 18.0	B	152.3 ± 43.8	A
Ea110 Surround, 1%	202.7 ± 28.7	B	188.9 ± 14.3	A
Ea110 Surround, 2%	185.5 ± 17.2	B	178.9 ± 15.8	A
Ea110 Surround, 4%	83.2 ± 43.4	C	115.3 ± 33.7	B
Ea110 Surround, 8%	49.3 ± 25.1	C	67.3 ± 9.7	C

UVA- Over the course of six hours, the PFU of $\Phi 21-4$ was not reduced. To investigate whether it was the bacteriophage not being affected by the UV or if the lights were not emitting correct wavelengths, an additional bacteria, *P. chloraphis* 06, was tested under the same UVR conditions for 6 hours. Within this timeline there is a steady loss of CFU, suggesting the UV light is functioning correctly (Fig. 15).

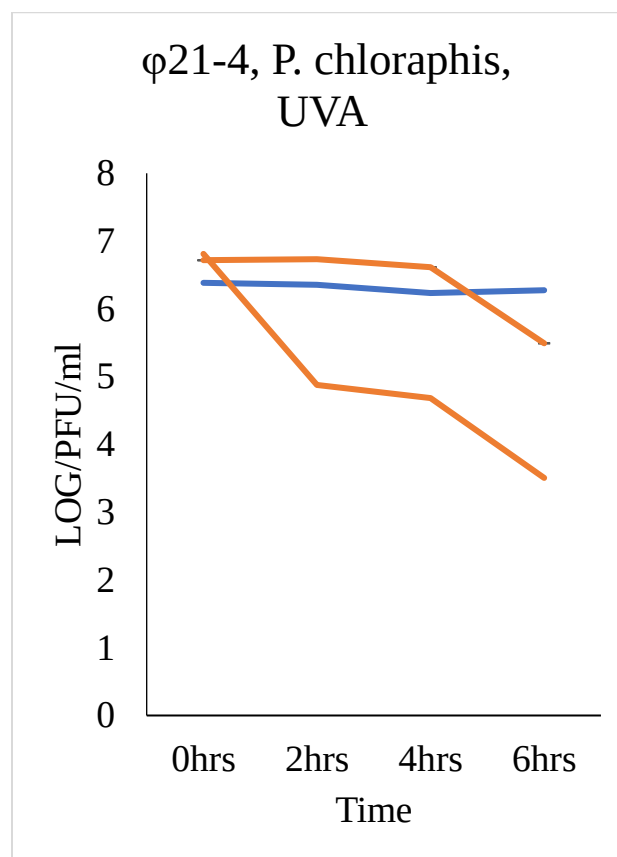


Figure 15: $\Phi 21-4$ and *P. chloraphis* exposed to UVA $\Phi 21-4$ (blue), *P. chloraphis* (orange), tested under UVA. The *P. chloraphis* was tested twice for consistency.

DISCUSSION

When exposed to UVR, specifically UVB and UVC wavelengths, bacteriophage survivability is lost very quickly. However, when UV-protectants, peptone and kaolinite clay, are added to the bacteriophage, even at lower concentrations, the bacteriophage are protected from significant degradation over the course of prolonged UVR exposure.

When conducting the UVA experiment, it was noted that very little killing of both the bacteria and the bacteriophage occurred. A representative curve was produced to visualize the subtle yet noticeable bacterial population and its constant curve. When an additional bacterium, *P. chloraphis* 06, was tested, a curve was developed, showing a reduction in bacterial population over the course of exposure. The reduction of this UVA-sensitive bacterium but not the bacteriophage and *E. amylovora* Ea110 suggests that UVA radiation is not causing DNA damage to the bacteriophage. Thus, in the environment, it is likely that only solar UVB radiation is impacting the survival of bacteriophage Φ 21-4 and Φ 31-3.

During the exposure to UVB radiation, both 8% Surround and peptone at 10 mg ml⁻¹ provided the greatest amount of UV protection. With the reduction of the concentration of the UV- protectant there is a direct correlation with the reduction in the amount of PFU. However, while the lower concentrations of UV-protectants, 5 mg ml⁻¹, 1% and 2% Surround, do not have as superior protective qualities as the greater concentration UV- Protectants, 8% and 10 mg ml⁻¹, the lower concentrations demonstrate a level of protection that is still a superior alternative to not using any form of UV-protectants. Lower concentrations were utilized in this experiment to signify the economic savings that can be achieved by fruit producers, while also achieving adequate bacteriophage protection. As an applied, extension-based research project, working for

the producers and helping the fruit tree community achieve greater disease protection at a minimal cost is a top priority.

When bacteriophage are exposed to UVC radiation there is significant loss in PFU as this is the most intense UVR. While UVC does not penetrate the atmosphere, it is still valued for *in vitro* experiments. The efficacy of both peptone and kaolinite clay is most likely due to the viscosity of the substance and bacteriophages ability to bind to clay particles (Vettori et al., 2000). For the peptone trials, 50 mg ml⁻¹ was tested, along with smaller amounts at 10 mg ml⁻¹ and 5 mg ml⁻¹. By reducing the amount of UV-protectant we were able to visualize optimal protection with minimum product as the 10 mg ml⁻¹ and 5 mg ml⁻¹, while not as effective as 50 mg ml⁻¹ peptone, still demonstrated protective qualities, while optimizing the economic impact. The Surround (kaolinite clay) trials, which were diluted to 8%, 4%, 2%, and 1% the recommended application rate, showed that even at 8% and 4% the recommended rate, superior protection can be upheld. As demonstrated by Vettori et. Al, (2000), we were also able to prove, that the addition of kaolinite clay to bacteriophage is significantly better at preventing inactivation of bacteriophage and preventing a reduction in PFU's.

CHAPTER THREE:

RELATIONSHIP BETWEEN BACTERIOPHAGE AND *E. AMYLOVORA* BACTERIAL POPULATIONS WHEN APPLIED IN AN ORCHARD SETTING

INTRODUCTION

The act of testing bacteriophage in both a lab, *in vitro* setting, as well as in a field setting is important for identifying any inconsistencies that may prevent the phage from performing at full capacity. Being able to model a field setting for research can allow greater credibility in the data produced and a reliability that the product being utilized by producers is working efficiently. Furthermore, a large quantity of bacteriophage studies are done predominantly *in vitro*. With the number of field setting experiments truly lacking. The first time bacteriophage were documented as being used as a biological control against fire blight was in 1973 (Erskine, 1973; Jones et al., 2013). The phage were isolated from soil surrounding an infected pear tree (Erskine, 1973). Later studies, conducted at Michigan State University, three bacteriophage were isolated from an apple orchard in East Lansing, Michigan. When applied at 10^8 PFU ml⁻¹ on flowers containing *E. amylovora* Ea110, the phage populations stayed constant, while those applied to flowers that had not been inoculated with *E. amylovora*, Ea110, the PFU dropped by half to 10^4 (Schnabel et al., 1999). It was concluded that for bacteriophage populations to be supported the bacterial host also had to be present. Phage research is not limited to the United States, in Canada, antagonist bacteria are used as carriers to transport the bacteriophage directly to the infectious bacteria (Jones et al., 2018; Svircev et al., 2018). In Hungary, it has been demonstrated that phage can penetrate seedlings and reduce the occurrence of fire blight (Koložsváriné Nagy et al., 2015). With the continuous fear of antibiotic resistance and a negative public perception, the need for more natural biological controls is necessary. However, the need for field trial experiments is

only rising. This allows for the suggestion that further research should be conducted in the field setting utilizing *Erwinia* bacteriophage and *E. amylovora*, Ea110. Further evidence supporting bacteriophage as a biological control agent will only concrete its importance in the fruit tree industry. This encouraged our replication of trials be done in a field setting to better analyze any variation among the results and for us to receive a clearer view of the prospective of using phage in an actual orchard.

MATERIALS AND METHODS

For this experiment, this field data is divided between the 2019 and 2020 seasons. In 2019, the field trials were divided into three main projects, two of them being bacterial population studies, and the third being a full-tree disease incidence study. The first bacterial population study was conducted by pipette inoculation of individual flowers, *E. amylovora* bacteria were concentrated to 10^5 CFU ml⁻¹. The second, 2019 experiment was done using a spray water bottle, the *E. amylovora* Ea110 bacteria was concentrated to 10^5 and suspended in spray bottles for a total volume of 500ml.

The full-tree treatments tested the efficacy of current commercial bacteriophage products as well as some still in trial, combined with potential UV-protectants. The purpose of this experiment was to mimic an orchard that had been infected with fire blight and sprayed with various forms of bacteriophage and protectants to determine how efficient each combination was at reducing the overall disease incidence. Treatments consisted of the commercial Certis bacteriophage cocktail, Agriphage, combined with various protectants such as carrot juice and peptone. Other treatments were FireQuencher A and B, a cocktail currently still in trial use, and Φ21-4, and Φ31-3, with various protectants.

The 2020 bloom experiment varied slightly from the 2019 bloom experiment. Due to the COVID-19 Pandemic, getting resources, such as bacteriophage, became an issue and forced the experiment to change. The 2020 bloom experiment centers around two population studies. For each of these experiments there were 12 treatments. The first six treatments (1-6) and second six treatments (7-12) were identical, however, flowers in the second set of six treatments (7-12) received an additional application of treatments, to determine if there was a potential second decrease in CFU of the bacterial population. Treatments consisted of Agriphage mixed with peptone and Surround, as well as a water control. *E. amylovora* Ea110 bacteria was concentrated to 10^6 and applied to designated Kit Jon and Golden Delicious trees using a backpack sprayer. The treatments were applied following the bacterial application. After 48 hours, the second treatment application was applied to treatments 7 through 12.

2019; Field Experiment I:

On the first day of 70% bloom, in the Gala block at the Horticulture Farm on Michigan State University Campus located in East Lansing, MI. *Erwinia amylovora* Ea110 bacteria was pipetted in 5 μ l increments onto the floral stigma clusters of 30 total flowers for each of the nine treatments. These treatments consisted of three phage treatments, three phage treatments with an added UV protectant, and three controls. After the bacteria had been administered and given adequate amount of time to dry the individual treatments were applied using a water bottle that contained a total volume of 500 ml. When sampling, single stigma clusters were placed into small glass tubes containing 1ml of Phosphate Buffer Solution (PBS). After the first sample, the treatments were applied. The remaining samples were taken at 16 hours AI (after inoculation), 24 hours AI, and 48 hours. AI, for a total of four samples. Back at the lab, the tubes containing the stigma clusters were sonicated before being serially diluted. From these serial dilutions, three, 25

µl spots were plated for each dilution onto LB agar plates containing rifampicin and cycloheximide. The plates were left in the 27 degree C Incubator for 24-48 hours until *E. amylovora* colonies were visible to count and record.

2019; Field Experiment II

For the second bacterial population study, *E. amylovora* Ea110 bacteria concentrated to 10^5 were suspended in spray bottles for a total volume of 500ml. Treatments of bacteriophage with UV-protectants were mixed in water bottles and sprayed onto flower clusters. Similarly, to the pipette inoculation, the first sample was taken after the *E. amylovora* inoculation and before the treatment applications. Samples were taken at time point 0, with no treatment application, 16 hours AI, 24 hours AI, and 48 hours AI. The first three samples were collected in 25 ml size glass tubes containing 12.5 ml of PBS. A total of five stigma clusters were collected for each treatment at each sample time point. For the fourth sample the amount of PBS in each tube was reduced to 5ml to achieve better bacterial counts and accuracy. These samples were processed the same as the pipette inoculated samples.

2019; Full-tree experiment analyzing fire blight incidence

For this experiment, *E. amylovora* Ea110 was sprayed onto the trees before the treatments using backpack sprayers. Once the *E. amylovora* Ea110 had dried, the backpack sprayers were mixed with the treatment and two gallons of water. Each treatment was sprayed once, at 80% bloom, onto four separate trees, then again at full flower bloom. After the second spray the trees were left to later be monitored and rated for total disease occurrence.

2020; Full Tree experiment analyzing fire blight incidence

At 4pm on the first day the bacteria were mixed in a backpack sprayer with two gallons of water and was applied to all the designated flowers. Sample one was taken after the bacteria had dried. Five stigma clusters were collected into small glass tubes 15 ml tubes containing 5ml PBS. Treatments were applied using a water bottle with a total volume of 500 ml. The remaining samples were taken at 16hours AI, 24 hours AI, 48hours AI, and 72 hours AI. After the fourth sample, at 48 hours, treatments 6-12 had a second application of treatments. This was repeated for both the Kit Jon (Fig. 19) and Golden Delicious (Fig. 20) cultivars. Once collected, the samples were processed the same as in 2019.

For the Golden Delicious cultivar, due to timing, and the weather progressing very quickly, several of the treatments matured at such an accelerated rate the *E. amylovora* bacteria was no longer viable or producing countable bacterial colonies. For this experiment at sample 4 and 5, samples were only taken from treatments 2- Agriphage with 5 mg ml⁻¹ peptone, water, Agriphage alone, and treatment 10, also water. These four treatments demonstrated continuous bacterial counts while the others did not produce initial bacteria, thusly, Eliminating those treatments from the experiment.

RESULTS

The results for the population dynamics, experiment I (Fig. 16) varied between the treatments. Each treatment had initial CFU between 2.5 to 4 logs. By 16hours AI, all the treatments except for the controls of water and 5mg ml⁻¹ peptone, and 31-3 with 5 mg ml⁻¹ peptone had a noticeable reduction in the CFU. Such that a minimum of half a log of CFU was

reduced in treatments (Φ 21-4 and Φ 31-3, Φ 21-4 with 5 mg ml⁻¹ peptone, and Φ 31-3 alone). By 24 hours AI, other treatments reached the lowest bacterial CFU for the experiment (Φ 21-4 and Φ 31-3 together, Φ 21-4 and Φ 31-3 with 5 mg ml⁻¹ peptone, 31-3 with 5 mg ml⁻¹ peptone, Φ 31-3 alone). Between 24 and 48 hours every treatment, except for water, saw an upward curve of increase in CFU's.

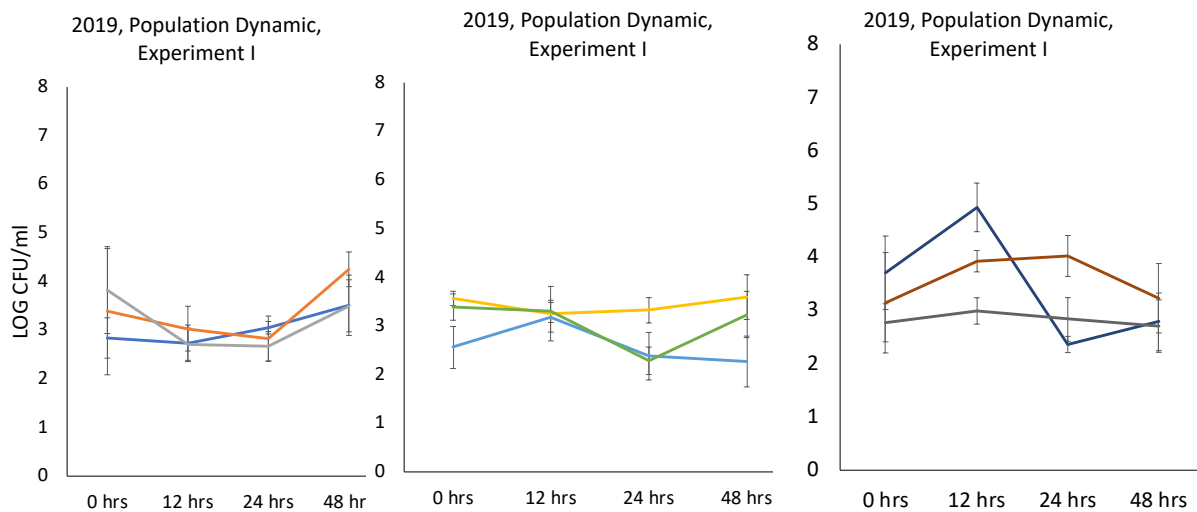


Figure 16: 2019 field study, Population Dynamics, Experiment I *Effects of natural UV irradiation on a total of nine treatments across a 48-hour time frame. (Left) Φ 21 (blue), Φ 31 (orange), Φ 21, Φ 31 (grey), (Middle) Φ 21, 5 mg ml⁻¹ peptone (yellow), Φ 31, 5 mg ml⁻¹ peptone (blue), Φ 21, Φ 31, 5 mg ml⁻¹ peptone (green), (Right) surfactant (dark blue), water (red), 5 mg ml⁻¹ peptone (grey).*

The second experiment, population dynamics experiment II, was only inoculated using a spray water bottle. It is evident from Fig. 17 that between the initial sample at time point 0 and the second sample at 16 hours, there was a rapid influx in CFU's, so much as up to 3.5 logs (Φ 21-4 and Φ 31-3 with 5 mg ml⁻¹ peptone). However, by 24 hours that increase had either plateaued (Φ 21-4 and Φ 31-3, Φ 21-4, and Φ 31-3 with 10 mg ml⁻¹ peptone) or drastically declined (Φ 21-4 and Φ 31-3 with 5 mg ml⁻¹ peptone, and kasugamycin). By the 48 hours AI sample, the

control, water, and $\Phi 21$ -4 and $\Phi 31$ -3 with 5 mg ml^{-1} peptone, demonstrated a steady increase in CFU's.

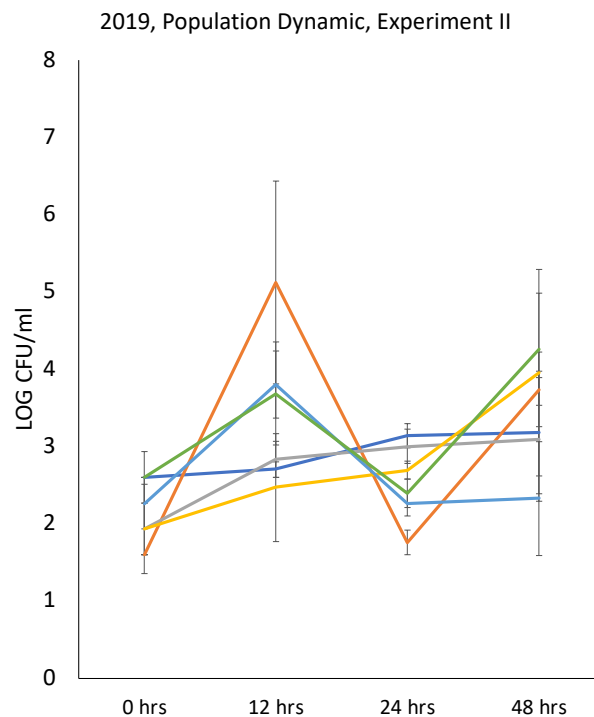


Figure 17: 2019 field study, Population Dynamics, Experiment II *The effects of natural UV irradiation in a field setting on six different treatments across 48 hours. Inoculation occurred using a spray bottle. Treatments are as follows, $\Phi 21, \Phi 31$ (dark blue), $\Phi 21, \Phi 31, 5 \text{ mg ml}^{-1}$ peptone (orange), $\Phi 21, \Phi 31, 10 \text{ mg ml}^{-1}$ peptone (grey), Water (yellow), kasugamycin (light blue), surfactant (green).*

Fire blight incidence was rated at the blossom blight stage and was based off of the percentage of blossom blight in 150 clusters per tree. This was replicated four times for each treatment (Fig. 18). The untreated control of water resulted with 67.7% blossom blight incidence. Incidence of blossom blight was better controlled with addition of treatments. The treatment with the greatest control of only 27.5% blossom blight incidence was a 4qt/100-gal application rate of Agriphage. The treatment with the least amount of control at 50.7% is also Agriphage, but with the addition of 10% carrot juice. The third Agriphage treatment had the addition of 5mg/ml of peptone and showed nearly 60% control with only 36.7% disease incidence. Other treatments,

such as the FireQuencher B had 34% disease incidence while the combination of Φ21-4 and Φ31-3 had 43.3%. While these two are numerically different, they are not significantly different ($P < 0.05$). FireQuencher A, while not significantly different to the Agriphage with carrot juice at 50.4% incidence, the addition of 5 mg ml^{-1} peptone to FireQuencher A allowed the disease incidence to drop to only 47% blossom blight. Finally, the last treatment of Φ21-4 and Φ31-3 with 5 mg ml^{-1} peptone demonstrated significant disease protection by only having 29.5% blossom blight incidence. An additional treatment of Φ21-4 and Φ31-3 with 10 mg ml^{-1} peptone was applied, however, due to field error the data is not being presented as valid.

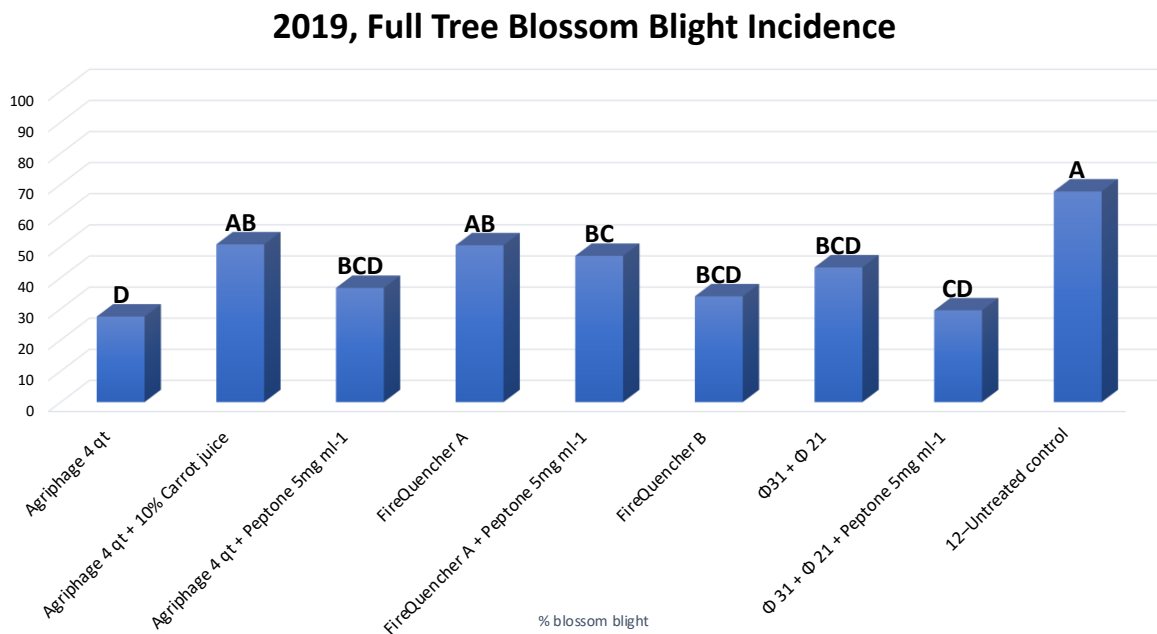


Figure 18: 2019 Full Tree Blossom Blight Incidence Twelve different bacteriophage treatments were tested. From left to right, Agriphage, Agriphage + 10% Carrot juice, Agriphage + 5 mg ml^{-1} peptone, FireQuencher A, FireQuencher A + 5 mg ml^{-1} peptone, FireQuencher B, Phage 31 + Phage 21, Phage 31 + Phage 21 + 5 mg ml^{-1} Peptone, Untreated control.

The 2020 Kit Jon population dynamic experiment demonstrated trends for all 12 treatments (Fig. 19) Treatments 1 through 6 expressed a control of a reduction in the overall CFU's within the first 24 hours AI. However, at the 48-hour AI mark, all every treatment, except for two (5 mg ml⁻¹ peptone, and Agriphage with 5 mg ml⁻¹ peptone), expressed a rapid incline in CFU's. For treatments 7 through 12, similar controls or constant CFU counts were demonstrated for the first 24 hours AI, then, similarly to treatments 1 through 6, the CFU count quickly increased, upwards of two logs in two treatments (Agriphage with Surround and water). Then, once the second application of treatments were applied on treatments 7 through 12, three of the six treatments showed a reduction in CFU's, which is not seen in the first treatments that did not have a second application. The three treatments that did not result in a reduction in CFU after the second application were, Water, Agriphage and peptone, and 5 mg ml⁻¹ peptone. Which 5 mg ml⁻¹ peptone, did not show a progressive curve at any time in the 72 hours AI.

The 2020 Golden Delicious, population dynamics (Fig. 20), conducted the same as the Kit Jon experiment, where treatments were applied after the 0 hours AI sample, and once again after the 48-hour AI sample for treatments 7-12. However, due to the Covid-19 pandemic and progression of the bloom season, only four treatments were able to be conducted to the full 72-hour AI sample. Treatment 2- Agriphage + 5 mg ml⁻¹ peptone, showed a steady progression and increase of CFU's up to the 48-hour AI sample, where it drops. Treatment two did not receive a second treatment application at 48 hours AI. Treatment 4- Water, trended upwards in progression of CFU's. Treatment 7- Agriphage, demonstrated an increase in CFU's until 16 hours AI, where it decreased in CFU's a single log. At 48-hour AI the curve trended upwards once more. Finally, treatment 10 -Water, showed a continuous upwards trend.

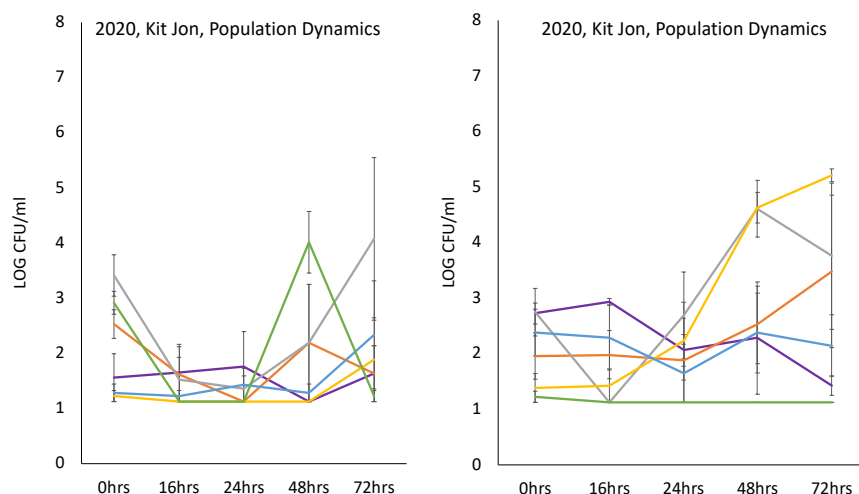


Figure 19: 2020 field trials, Kit Jon Population Dynamics *Bacterial population study conducted in 2020 on Kit Jon Apple trees, (Left) graph contains treatments 1-6, Agriphage (purple), Agriphage+5 mg ml⁻¹ peptone (orange), Agriphage + Surround (gray), Water (yellow), Surround (blue) 5mg ml⁻¹ peptone (green), respectively. (Right) Treatments 7-12, Agriphage (purple), Agriphage+5 mg ml⁻¹ peptone (orange), Agriphage and Surround (gray), Water (yellow), Surround (blue) 5 mg ml⁻¹ peptone (green), respectively. Treatments 7-12 were applied a time at 48 hours AI.*

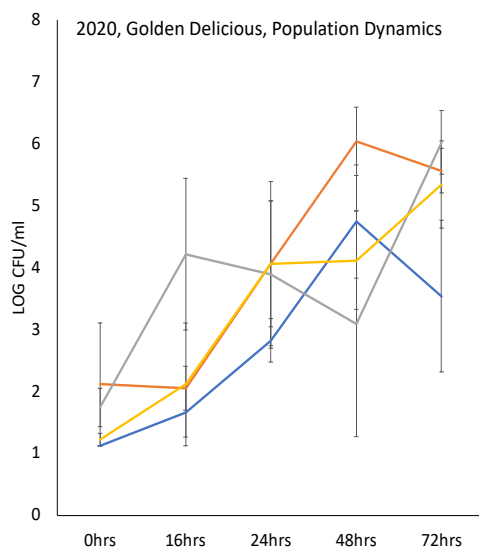


Figure 20: 2020 field trials, Golden Delicious Population Dynamics *Bacterial population study conducted in 2020 on Golden Delicious apple trees. Due to unforeseeable events of the 2020 Covid-19 pandemic, only four treatments were able to be fully conducted to 72 hours. Treatment 2- Agriphage + 5 mg ml⁻¹ peptone (blue), Treatment 4- Water (orange), Treatment 7- Agriphage (gray), Treatment 10 -Water (yellow). Treatments 7 and 10 were applied a second time at 48 hours AI.*

DISCUSSION

The 2019 and 2020 field season suggests that, depending on the bacteriophage and its combination with UV-protectants, bacteriophage can adequately protect fruit trees from a fire blight infections. One of the most common trends that is demonstrated within Fig. 16 and 17, shows a reduction in the overall CFU up until 24 hours. This suggests that bacteriophage are most active at lysing bacteria between 16- and 24-hours AI. However, after the 24-hour time point there is a generally a large incline in the CFU. It is probable the bacteriophage are at their highest activity within 24 hours, then the ratio of bacteria to phage is no longer adequately balanced for proper lysing. This encouraged the second application of treatments in the 2020 field season (Fig. 19 and 20). It was hypothesized that, by applying a second application of treatments at 48 hours, there is a potential advantage to get ahead of the next spike in bacteria population. The addition of multiple applications allows for the assumption that phage will continue to be active and will continue to lyse bacteria. However, it is highly unlikely to be able to pin-point a specific hour or time range of when phage are lysing (Kering et al., 2019). Thusly, supporting the case that multiple applications will provide the best protection. In 2020, the field season results vary from what would be expected. For the Kit Jon's (Fig. 19), the addition of a second treatment application proved to have a positive effect in reducing the bacterial CFU's. while in the Golden Delicious trials (Fig. 20), very little control was ever obtained, even with a second application of treatments. However, this could be due to the experiment starting so late in the bloom period, the flowers were past the time of peak susceptibility.

The addition of UV- Protectants to bacteriophage in a field setting has the ability to reduce the overall disease significance, especially when a second application of bacteriophage is applied at the 48 hours AI time point. Furthermore, the addition of Surround as a UV-protectant

demonstrated bacteriophage protective qualities. Surround, which is currently an OMRI (Organic Material Review Institute) listed product for use on fruit trees, grapes, and vegetable crops as a deterrent to insects. It is also labeled as a barrier product that will reduce UV exposure and reduce the temperature that the plant is exposed to (OMRI, 2000). Because of these protective qualities, and Surround being readily available, our group believed it had great potential as a UV- protectant. From these experiments we saw that the addition of peptone and Surround to be effective in protecting bacteriophage from UVR degradation.

FUTURE DIRECTIONS

Moving forward, it is important that the field studies be conducted at least one more year to determine if there are any consistencies, as both 2019 and 2020 field results varied so much from each other. Conducting the 2020 experiment again with a second application of treatments at the 48-hour AI timepoint is a good direction to explore more. I believe returning to the pipette inoculation technique used in population dynamics experiment I in 2019 would be the best option as we observed greater accuracy among the treatments. I do not think the water bottle inoculations were as effective at creating a bacterial population as we hoped they would. Furthermore, *in vitro* research on the effects of UVA irradiation on bacteriophage could show promising results as 95% of the atmosphere is UVA. This leads to question as to why bacteriophage quickly degraded in a natural, field setting, but did not under high UVA irradiation in a lab setting. There could be several factors that contribute to this such as weather, susceptible host presence, and time of day. Finally, like the antagonist bacteria research that was conducted in the 1990s (Stockwell et al., 1996), it would be interesting to see how bacteriophage work alongside antibiotics as a supplementary bacterial control in both an *in vitro* as well as field setting.

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