Comparing the effect of various cations on bacteriophage activity against *Xanthomonas euvesicatoria*

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Abstract

Bacterial Spot is a common plant disease that affects tomatoes and peppers grown worldwide. This disease is caused by four phytopathogenic bacterial strains belonging to the genus *Xanthomonas*: *X. euvesicatoria*, *X. perforans*, *X. vesicatoria*, & *X. gardneri*. Copper-mancozeb bactericides – the current standard for controlling the spread of Bacterial Spot – are being used continuously to combat emerging copper-resistant *Xanthomonas* strains. A bacteriophage biocontrol was proposed as an alternative approach for controlling Bacterial Spot and preserving crop sustainability. The research objective was to investigate methods for enhancing bacteriophage virulence, while minimizing the negative non-target environmental impacts associated with copper-mancozeb use and existing bacteriophage biocontrol formulations. This study aimed to implement the plaque assay approach utilized in our previous calcium supplementation study, to test and compare the effect that K^+, Na^+, Zn^{2+}, and Mg^{2+} supplementation, at five different concentrations, have on our purified bacteriophage samples against our previously identified bacterium, *Xanthomonas euvesicatoria*. We hypothesized that supplementing Phages FFC-3, FFC-8, and FFC-11 with these divalent and monovalent cations will yield an effect on virulence activity against the *Xanthomonas* phytopathogen, and that cation supplementation at concentrations ≤ 0.1mM will induce a more significant degree of phage virulence than at concentration > 0.1mM. Plaque assay results showed that divalent cation supplementation had a greater effect on phage virulence than monovalent cations. Larger, clearer, and more abundant plaques were mostly observed in phages supplemented with cations at concentrations ≤ 0.1mM. This study will address uncertainties regarding cation-phage interactions when tested against the growth of a phytopathogenic bacterium.

Introduction

Sustainability Issue

To meet the food demands caused by global overpopulation, it is imperative for crop supply to increase by as much as 80-110% [8,39]. However, to reach that level of production and sustainability, the impact of crop disease must be reduced. In fact, it has been previously estimated that at least 10% of global food production is lost to plant diseases [8,43]. One such plant disease, known as Bacterial Spot, is common everywhere in the world where tomatoes and peppers are grown, and is especially severe in the southeastern region of the United States due to the high temperature, high humidity, and rainy climate [44]. In the case of severe Bacterial Spot infections, about 23%–44% of direct tomato and pepper crop yield losses can be experienced per year [7,34]. Bacterial Spot is caused by the following four aerobic, gram-negative species of phytopathogenic bacteria belonging to the genus *Xanthamonas*: *X. euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri*; all of which were formerly grouped together and referred to as *Xanthomonas campestris pv. vesicatoria* [23,44].

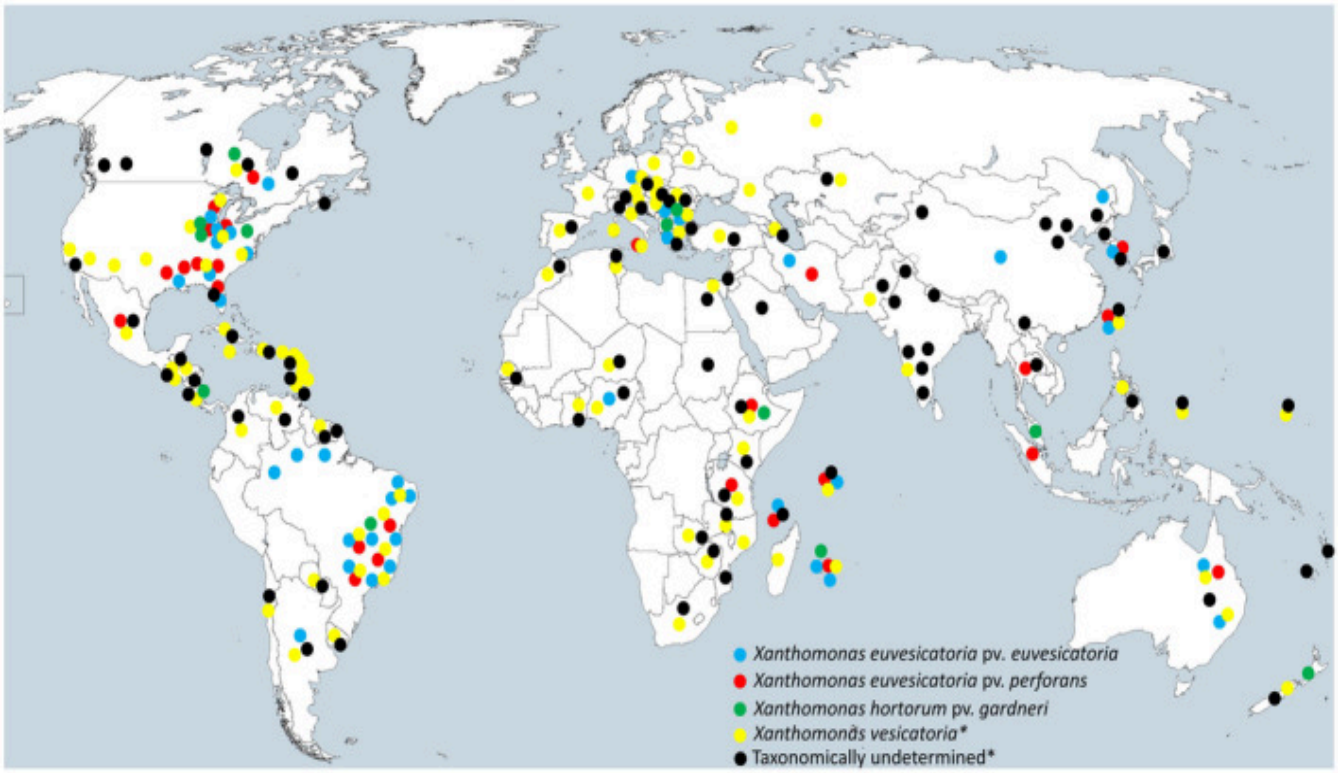

Figure 1: Geographic distribution of Bacterial Spot disease causal agents. This image highlights the wide-spread nature of this plant disease, as it can affect tomatoes and peppers anywhere in the world that they are grown – regardless of climate. The distribution of taxonomically undetermined Bacterial Spot phytopathogens indicates that these bacteria continue to evolve novel strains, which may lead to the acquisition of more resistance mechanisms against current treatment standards [34].

Typically, plants are resistant to non-specific pathogens due to the presence of a waxy cuticle covering the epidermal cell layer and the constant synthesis of various antimicrobial compounds. However, phytopathogens, such as members of *Xanthomonas campestris pv. vesicatoria*, require either the presence of damaged tissues, specialized structures (e.g., stomata) for entering the cell, or a specific carrier (vector) to penetrate plants, which often renders the mechanisms of host protection ineffective [32]. Species and pathovars of the *Xanthomonas* genus typically exhibit a high degree of host- as well as tissue-specificity, invading either the xylem or intercellular spaces of the mesophyll parenchyma tissue of the host plant [8,40]. These bacteria can enter through natural openings, such as stomata and hydathodes, and wounds created by environmental factors, such as wind-driven sand, insect punctures, or mechanical means [21,44]. Furthermore, the bacterium can be recovered from non-symptomatic plants in as early as one week at distances of up to 4 m from neighboring symptomatic plants [44]. Symptoms of Bacterial Spot disease generally present themselves as lesions that are dark brown or black in color and circular-shaped (but can be angular) appearing on any aboveground plant parts, such as the leaves, stems, and fruits [44]. The environmental sample collection phase of this study involved the recognition of Bacterial Spot disease symptoms on an infected tomato plant in the field, to acquire leaf, soil, and irrigation water samples. These samples were then used to isolate one of the four phytopathogenic members belonging to *Xanthomonas campestris pv. vesicatoria*. Copper-based bactericides, which are the current standard for controlling the spread of Bacterial

Spot disease, have begun to impact the environment negatively, and decrease in effectiveness against *Xanthomonas* bacteria.

Bacterial Resistance

Since the early 1800s, copper has been used as a treatment to manage plant pathogens, such as *Xanthomonas campestris pv. vesicatoria*. When solubilized in water, copper ions bind tightly to sulfhydryl groups, accounting for its biocidal properties [44]. However, by the 1980s, strains of this phytopathogen exhibiting tolerance to copper emerged, which limited the effectiveness of copper-based bactericides [30,44,46]. Thus, control of Bacterial Spot disease was only achievable with the addition of an ethylene-bis-dithiocarbamate compound known as mancozeb, to the copper bactericide. Since the emergence of copper-resistant *Xanthomonas* strains in the 1980's, copper-mancozeb treatment mixtures have been the standard practice for controlling bacterial spot [10,30,44]. However, there are many disadvantages that result from the excessive use of this method to combat the increasingly resistant phytopathogens. Copper-mancozeb bactericides can cause negative non-target environmental impacts. For instance, high concentrations of copper can be toxic to the afflicted plant, as well as the surrounding flora (phytotoxicity); overaccumulation of copper ions due to their inability to degrade in the soil, can cause aridity (which reduces soil quality for cultivation) [25,44]; and the continuous decrease in copper effectiveness against resistant pathogens [44]. Since copper-mancozeb fails to adequately control Bacterial Spot when copper-tolerant strains are present under optimal disease conditions [22,33,44], we proposed a plant biocontrol method implementing bacteriophages as an alternative approach for controlling the spread of this disease.

Bacteriophage Biocontrol

Bacteriophages are viruses that can infect and kill bacterial cells without any negative effect on human or animal cells [36]. They can be found in every environment where their bacterial hosts are present [11]. Phages are highly specific, with most infecting only a single species of bacteria. These viruses can exist in either a virulent (lytic pathway) or temperate (lysogenic pathway) state (Figure 2). Virulent phages undergo the lytic cycle following attachment and penetration of the bacterial host cell for injection of its genetic material (*Xanthomonas*-specific bacteriophages are DNA viruses). In the Lytic pathway, the viral DNA undergoes transcription and translation with the aid of the host's cellular machinery in order to synthesize viral proteins. These proteins are then assembled into new virions (infectious phage cells) until the host cell lyses (ruptures) and releases the phage progeny into the environment until the cycle is repeated. On the other hand, a Temperate phage can also follow the lytic route of infection but initially follows the route of lysogeny, where the phage genome integrates into the bacterial chromosome (to form a prophage) or persists as a plasmid [8]. Furthermore, the viral DNA of a Temperate phage can only separate from the host genome and initiate the lytic cycle under conditions that are unfavorable for the host cell (i.e., cellular stress). For attachment onto the surface of a bacterial cell, bacteriophages bind to specific receptors on the surface of the host cell. This specificity of interaction between phage attachment structures and host cell surface receptors, is the factor that influences the bacterial host range [11,45,49]. Since the isolated phytopathogenic bacterial strain was confirmed to be *Xanthomonas euvesicatoria*, according to the preliminary results of the current study, we predicted that the species-isolated bacteriophage samples most likely belonged to the *Myoviridae* family [12,13]. Members of the *Myoviridae* family are characterized by contractile

tails/sheaths, structural rigidity, and an icosahedral capsid head, and may attach to the surface components of bacterial cell walls in *Xanthomonas* bacteria, such as lipopolysaccharides (LPSs) and lipooligosaccharides (LOSs) [24,42].

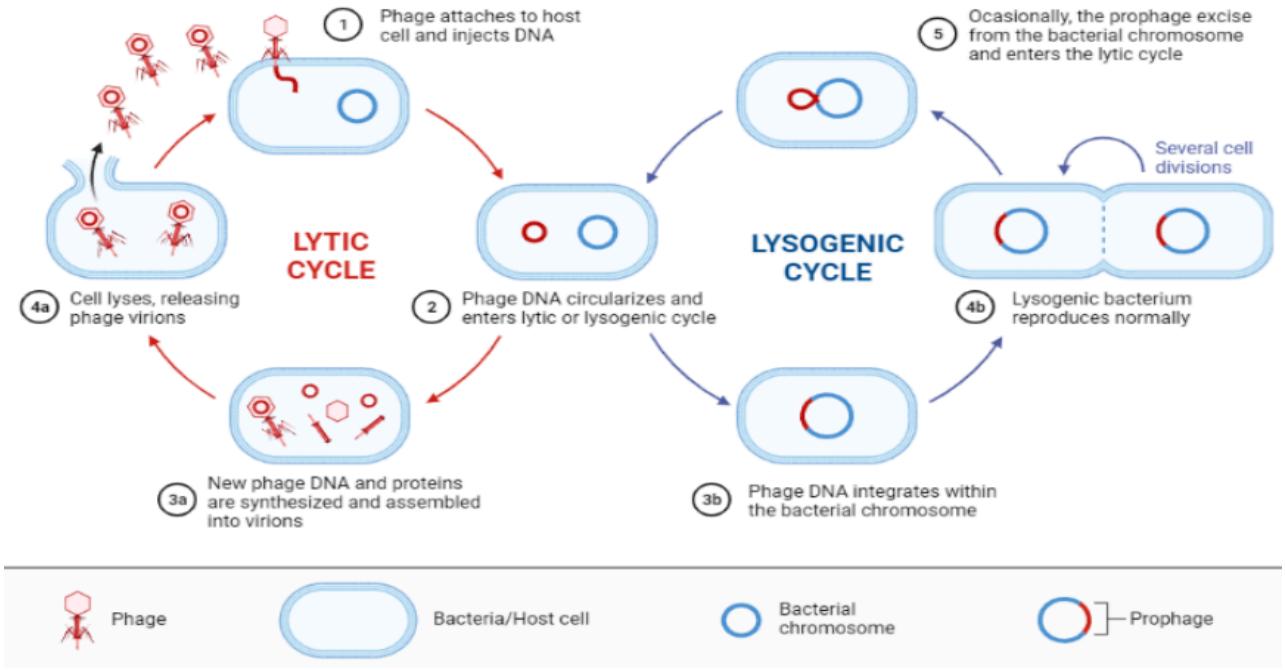

Figure 2: Diagram depicting the lytic and lysogenic cycles of bacteriophages. Bacteriophages can either be Virulent (infectious) or Temperate (dormant). Only Temperate phages are able to transition from the Lysogenic pathway to the Lytic pathway when the bacterial host is under conditions of cellular stress. New phage cells are synthesized and released via the Lytic cycle – resulting in the lysis of the host cell [36].

Unlike chemical biocides, phages occur naturally in the environment and humans are therefore exposed to them frequently without any harm [8]. After application of a phage treatment *in vivo*, the number of virions (infectious phage particles) increase if the respective bacterial host species remains accessible. However, phages can only persist in high numbers in any environment if the host is present [8,20]. Another potential disadvantage is that the bacterial hosts themselves may develop mechanisms by which they can inhibit the process of phage propagation, such as preventing phage adsorption, preventing DNA integration by Superinfection

exclusion system (Sie), degradation of phage DNA by Restriction-Modification (RM) defense system and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), and blocking phage replication, transcription, translation or virions assembly by Abortive Infection system (Abi) [11]. Regardless of these obstacles for a phage biocontrol treatment against phytopathogenic bacteria, the abundance of environmental phages provides a boundless opportunity to isolate and develop phage preparations against many current and future bacterial pathogens regardless of resistance mechanism development [14,49].

Minimizing negative non-target environmental impacts

Despite the potential advantages that the proposed bacteriophage-based biocontrol method may have over copper-mancozeb bactericides in controlling Bacterial Spot disease, ingredients found in current bacteriophage biocontrol formulations may result in collateral damage to the environment if adopted as the new standard. A biotech company based in Sandy, Utah, called OmniLytics, developed a product line called AgriPhage™ in 2005, which was the first commercialized and EPA-registered biopesticide implementing bacteriophages for the control of plant diseases such as Bacterial Spot of tomatoes and peppers [37]. OmniLytics claims that their AgriPhage™ formulation prevents the growth of *Xanthomonas* phytopathogens, and does not adversely affect plants, animals, humans, or other beneficial organisms [37]. However, when examining the list of ingredients in founding the AgriPhage™ product formulation, an ingredient of concern was identified. Aside from the inclusion of phages, cation sources, and nutrient sources, AgriPhage™ also contains silicone oil (α-Methyl-ω-methoxypolydimethylsiloxane) [1]. Although silicone oil functions as a penetrant (to improve foliar absorption of the biopesticide solution) and is widely used in pesticide production,

the inclusion of this substance in a bacteriophage biocontrol formulation may have negative consequences on the environment regardless of its intended effect [2]. According to the Safety Data Sheet information for this ingredient, silicone oil is toxic to aquatic life and is accompanied by long lasting effects [41]. Furthermore, the SDS presents a precautionary statement on the use of silicone oil – advising to avoid release of the substance into the environment, collect any spillages, and to dispose of this substance in an approved waste disposal plant [41]. Also, recent field trials showed that AgriPhage™ applications were not effective in reducing Bacterial Spot disease compared to copper sprays or nontreated plants [3,44]. Furthermore, the cost and inconsistent efficacy of commercially available bacteriophages pose a significant drawback for adoption into a spray program [44]. This could be considered a reason as to why a reliable alternative for the current copper-mancozeb standard has not yet been adopted by tomato and pepper farmers in recent years. Therefore, this research aims to investigate methods for improving the effectiveness of bacteriophage biocontrol formulations in controlling Bacterial Spot disease more consistently, while attempting to eliminate any ingredients, such as silicone oil, that may result in negative non-target environmental impacts.

Implementation of Cations

Rather than engineering a phage cocktail (mixture of various types of phages) [11] or utilizing antibiotic compounds to control the spread of Bacterial Spot more effectively [44], our study aimed to supplement phytopathogen-isolated bacteriophages with various monovalent and divalent cations. This approach was chosen based on the crucial roles that the following cations play in plant biochemical systems: Ca^{2+}, $Mg^{2+,}$ Zn^{2+}, K^{+}, Na^{+}. The presence of some of these cations in existing bacteriophage biocontrol formulations also makes them promising choices for

testing and experimentation in the development of the proposed biocontrol method. Molecules involved in the biochemical functionality and viability of a plant cell may also serve to facilitate phage lytic activity against a phytopathogen host. In our previous study, a plaque assay approach was implemented to test the effect of calcium ion supplementation on the lytic activity of our five *Xanthomonas*-specific phage samples (FFC-1, FFC-2, FFC-3, FFC-8, and FFC-11) at 0.01mM and 0.1mM against *X. euvesicatoria*. This approach yielded results that demonstrated an increase in virulence activity for each calcium-supplemented phage sample – via the induction of plaque formation – when compared to the absence of calcium (no plaque formation). Calcium (Ca^{2+}) exhibits a dual function, both as a structural component of plant cell walls and membranes, as well as an intracellular secondary messenger involved in signaling nutrient availability and pathogenic invasion [47]. Therefore, if a plant were to lack enough calcium, the cell wall would be weakened, and structures such as root hairs or pollen tubes, would not be able to expand during tip growth [6,47]. A previous study tested calcium ion absorption in the *Bacillus subtilis*-specific phage, Phage 41C, have indicated that Ca^{2+} concentrations ranging from 0.1mM to 10mM fulfill the requirement for phage-cation attachment, with the most efficient absorption occurring at reduced Ca^{2+} levels [27]. The results from this study demonstrated that Phage 41C experienced an enhancement in virulence activity against *B. subtilis* when at least 10mM of calcium was present [27]. In the current study, the degree of phage lytic activity was examined *in vitro* and determined based on the presence, size, quantity, and turbidity (cloudiness) level of the plaques (clear circular zones absent of bacterial growth; indicator of phage virulence activity) formed after incubation of plaque assay dilution plates [38].

Each of the cations that were tested in this study have a significance tied to their selection. For instance, magnesium plays a central role in plant chlorophyll biosynthesis and

carbon fixation as a cofactor of a series of enzymes involved in carbon metabolism [15,18]. For zinc (Zn^{2+}), the outcomes of plant–pest/pathogen interactions vary, depending on the effectivity of the zinc-related responses in limiting the invader's attack as well as on the enemy's ability to circumvent the plant defenses, in addition to other environmental conditions that can favor either host or invader [9]. In fact, a protective zinc concentration against certain pathogens can also cause the plant to be susceptible to another pathogen on the same plant [9,17]. In the case of sodium, uptake of this cation at the root/soil boundary could assist in the phytoremediation of moderately saline soils [29]. Finally, potassium is an essential nutrient that plays a role in plant growth and metabolism and contributes to the survival of plants when exposed to various biotic and abiotic stresses [48].

Although there are currently no existing bacteriophage biocontrol formulations that solely implement environmentally safe cations, there are studies from the literature that test the effect of cations in phage-host infection systems. For example, a previous study conducted on the human pathogen, *Bacillus cereus,* tested the Optical Density (OD) of a *B. cereus* liquid culture in 1-hour increments (over 10 hours), in the presence of its isolated bacteriophages, BCP1-1 and BCP8-2 (added to the culture during Time Point 2), under multiple conditions of cation supplementation [5]. The following divalent and monovalent cations were added into the Nutrient Broth liquid culture medium, and each tested as a separate condition for each of the isolated phage samples: Ca^{2+}, Mg^{2+}, Mn^{2+}, and Na^{+}. The *B. cereus* growth curves that were generated for each condition implemented in this study, demonstrated that the growth of the B. cereus cells in a liquid culture with added cations, significantly decreased when its isolated bacteriophages were also present [5]. The findings also highlighted that cation-supplemented bacteriophages decreased the OD of the liquid culture in a shorter amount of time when compared to the control conditions

that tested *B. cereus* growth without cations or phage, and *B. cereus* growth in the presence of phage without cation supplementation [5]. These results are important to consider for the current study, as it hints at the potential of divalent and monovalent cation supplementation for increasing phage virulence activity against a bacterial host. However, despite these findings, little is still known about how bacteriophage interact with these cations in a phytopathogen host infection system. Furthermore, it is also important to consider the implications drawn from the preliminary results from our previous study, which demonstrated that more of our purified phage samples were able to yield plaques on the plaque assay plates containing 0.01mM Ca^{2+} (4 out of 5 phages) when compared to the plates containing 0.1mM Ca^{2+} (2 out of 5 phages).

Based on the implications from these findings, we proposed a continuation of the plaque assay approach implemented in our previous calcium supplementation experiment, for the current study. This approach will be used to test the effect that supplementation with the following divalent and monovalent cations: K^{+}, Na^{+}, Zn^{2+}, and Mg^{2+}, have on the virulence activity of our purified phage samples against *X. euvesicatoria*, at six different concentration levels (0mM, 0.001mM, 0.01mM, 0.1mM, 1.0mM, and 10mM). It was hypothesized that supplementation of Phages FFC-3, FFC-8, and FFC-11 with these cations will have an effect on virulence activity against the *Xanthomonas* phytopathogen. Furthermore, we hypothesized that cation supplementation of our phage samples will yield a more significant virulence effect against *X. euvesicatoria*, at concentrations $\leq$ 0.1mM, than concentrations > 0.1mM. This research will serve to investigate methods for improving the effectiveness of bacteriophages in plant therapeutic agents, as well as to minimize the negative non-target environmental impacts associated with existing bacteriophage biocontrol formulations and current copper-mancozeb Bacterial Spot treatment standards.

Materials & Methods

Environmental sample collection

To initiate this study, we purchased a healthy and fully-grown tomato plant from a Walmart in Parkland, FL. We then sowed the tomato plant on a patch of fertile soil in a partially shaded area located in the same city. The growth of the tomato plant was tracked over the course of three weeks, under uncontrolled environmental conditions, which was the time it took for symptoms of Bacterial spot disease to present themselves. Small, dark brown lesions were observed on the dorsal and ventral surfaces of the tomato plant leaves, in the presence of sunlight (daytime). Infected leaf samples were collected in a sterile 50mL conical tube and taken to the laboratory for further experimentation.

Isolation of the target bacterium

Once in the laboratory, a five-fold, 1:10 serial dilution was first performed on the acquired environmental sample. This was done by dispensing 10mL of 1.3% Nutrient Broth in the 50mL conical tube containing the sample, vortexing the tube to disperse the cells in solution, and then making five dilutions from that stock. The series of 5-fold dilutions were prepared by pipetting 100µL of the original inoculum (sample tube) into a 1.5mL Eppendorf tube containing 900µL of 1.3% Nutrient Broth, and then plating each dilution (10^{-1} – 10^{-5}), respectively, on Petri plates containing Tween agar medium. After incubation at 28°C for 96 hours, a mixed culture (multiple bacterial species) of single bacterial colonies formed on the surface of the dilution plates (Figure 1). Tween agar is a semi-selective medium that allows for the detection and isolation of *Xanthomonas* strains [31]. This medium consists of: 10g peptone, 0.25g anhydrous $CaCl_2$, 0.60g boric acid, 15g agar, and 10mL Tween 80 + 10mg of methyl green (added after

media is autoclaved) per 1L of de-ionized H_2O. Identification of the suspect *Xanthomonas* bacterium was aided by the Tween 80 in the medium, which is a fatty acid ester that acts as a substrate for lipolytic enzymes (enzymes that break down lipids) of the phytopathogen [31]. The release of fatty acids causes a white, crystalline precipitate to form in the surrounding areas of bacterial growth [31]. *Xanthomonas* bacteria are characterized by round, punctiform colonies with bright yellow pigmentation and a mucoid surface (Figure 2).

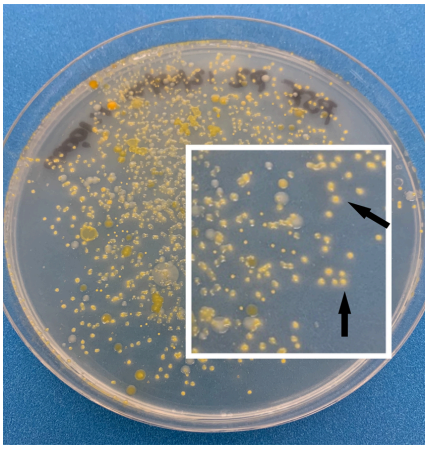

Figure 3: Mixed culture from environmental sample containing suspect *Xanthomonas* bacterium. 100µL of each dilution were dispensed and spread onto Tween agar plates and incubated for 96 hours at 28°C. After incubation, multiple single colonies of various bacterial species, grew on the surface of the five dilution plates. Suspect *Xanthomonas* colonies were detected based on colony morphology characteristics exhibited when reacting with the Tween 80 in the medium.

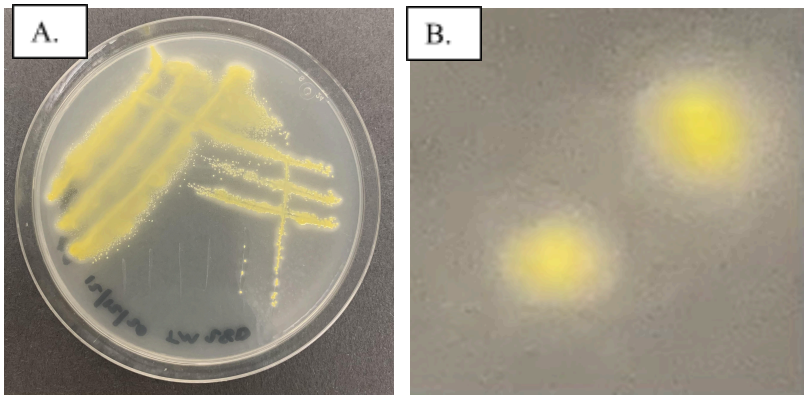

Figure 4: Pure culture of suspect *Xanthomonas* bacterium streaked on Tween agar medium. A suspect *Xanthomonas* bacterial colony from the previous mixed culture was picked with a sterile toothpick and streaked onto a Tween agar plate. After 48 hours of incubation at 28°C, the suspect *Xanthomonas* isolate grew on the surface of

the Tween agar medium (Image A). An enhanced view of the single colonies formed on the most dilute region of the streak plate (Image B). These colonies appear bright yellow in pigment with a surrounding halo containing a white, crystalline precipitate.

Characterization of the suspect isolate

Following its selective and differential isolation, the suspect *Xanthomonas* isolate was then plated on Nutrient Broth agar – a rich medium that favors its growth [44]. The suspect isolate was streaked onto the plate for isolation of single bacterial colonies, and incubated at five different temperatures (4° C, 25° C, 28° C, 30° C, and 37° C) for 72 hours. This confirmed that the suspect isolate grew most optimally at temperatures of 28°C–30°C. Next, a series of tests were conducted to verify other key characteristics of the target bacterium according to the literature. A Gram Stain was performed on the bacterial sample and confirmed that the suspect isolate was Gram-negative due to its pink coloration from retention of the counterstain [19]. A Sulfur Indole Motility (SIM) test was then performed, which demonstrated that the suspect exhibited motility (movement / locomotion) [19]. The bacterial sample was then plated on MacConkey agar – a medium that selects for the growth of Gram-negative bacteria and differentiates between the bacteria that ferment lactose and those that do not [19]. This test confirmed that the bacterial sample was Gram-negative because it was able to grow on the MacConkey medium. It also demonstrated that the suspect isolate was unable to ferment lactose because the pH indicator in the medium did not produce a pink coloration in the bacterial colonies. Lastly, a Plant Pathogenicity test and a Soft Rot test were performed *in vivo* – under non-controlled conditions – to observe whether the suspect isolate was able to induce Bacterial Spot disease symptoms on a healthy tomato plant sample. The Plant Pathogenicity test involved the utilization of a sterile cotton swab to pick a portion (observable amount) of the suspect isolate from a Nutrient Broth agar streak plate and smearing the bacteria onto the leaves of a healthy

tomato plant [26]. The plant was then left outside, and after about 2 weeks, medium-sized necrotic lesions (spots) appeared on the surface of the leaves at the zone of inoculation. Similarly, the Soft Rot (Fruit Pathogenicity) test involved the use of a sterile toothpick to pick a portion of the suspect isolate from a Nutrient Broth agar streak plate and puncturing the fruit of a healthy tomato plant [50]. The inoculated tomato fruit was then placed on a paper towel in a 28°C incubator and examined every 24 hours. After 72 hours of incubation, small necrotic lesions (spots) were observed on the surface of the tomato fruit at the zone of inoculation. Both, the Plant Pathogenicity and Soft Rot tests confirmed that the suspect isolate was able to induce Bacterial Spot disease symptoms in a healthy tomato plant, thus confirming its characteristics as a phytopathogen.

Identification of suspect isolate – Xanthomonas euvesicatoria

Once these tests confirmed the characteristics of the target bacterium, it was sent to the genome sequencing company, GENEWIZ, located in South Plainfield, New Jersey. The method used was rapid Sanger sequencing of the 16S rRNA gene sequence (a conserved phylogenetic marker commonly used in the identification of microorganisms). GENEWIZ then sent back the sequencing results demonstrating the percent similarity of the suspect isolate, which is represented by a percentage value generated from comparing the genome of the submitted bacterial isolate sample with every other mapped bacterial genome in the database. This confirmed that the genome of our suspect isolate was 99.4% similar to the genome of *Xanthomonas euvesicatoria*.

Bacteriophage amplification and isolation – Enrichment culture

To isolate the phytopathogen-specific bacteriophage samples, the number of phages present in our environmental samples were initially amplified by preparing an enrichment culture for each of the following samples: an infected tomato plant leaf, soil from under the infected tomato plant, and the water from the irrigation system used in the growth of the infected tomato plant [35]. To initiate the enrichment culture protocol, three 100mL flasks were first prepared for each environmental sample. 50mL nutrient broth, 0.5mL 100mM $MgCl_2$, 0.5mL 100mM $CaCl_2$, and 0.5mL of overnight *X. euvesicatoria* liquid culture, were then added to each flask. For the soil enrichment culture flask, 10g of the soil sample was added. For the plant tissue enrichment culture flask, 3g of the leaf sample was added. For the water enrichment culture flask, 10mL of the irrigation water sample was added. Each flask was then incubated at 28° C for 24 hours in a shaking incubator at 180rpm. After incubation, the bacteriophages present in each enrichment culture were isolated in separate 50mL conical tubes using a 0.22-micron syringe filter. The three phage filtrates prepared were then used to perform the subsequent Direct Spot test for the selection of the target phage samples.

Bacteriophage sample selection – Direct Spot test

Following the amplification and isolation of the phages present in the three previously collected environmental samples, a Direct Spot test was then performed for the purpose of selecting the most lytic phage samples – phages that form the largest and clearest plaques [35]. A plaque is a clear circular zone that appears on the surface of a confluent lawn and is absent of bacterial growth (key indicator of phage lytic activity against the host cell). The top agar recipe for the Direct Spot test includes the following: 4.5mL Nutrient Broth agar (nutrient broth+0.2% agar), 0.5mL 100mM $MgCl_2$, 0.5mL 100mM $CaCl_2$, and 200uL overnight *X. euvesicatoria* liquid

culture (added after top agar cooled to ~48°C following autoclaving at 121°C, 15 psig, for 15 minutes) per Spot assay plate. After preparing the 5.7mL combined top agar solution, it was then poured and overlayed onto a Nutrient Broth Agar plate labeled with a 6-box grid sticker. This was done for each of the 9 Spot assay plates used for this test. Once the top agar on each plate has solidified, 10uL aliquots of each mixed-species phage filtrate were dispensed onto the center of each of the 6 boxed sections on the respective Spot assay plate. Direct Spot tests of each of the three phage filtrates were done in sets of 3 (3 plates per filtrate for a total of 9 plates) and each plate in each 3-plate set was incubated at a different temperature to determine the optimal plaque-forming temperature for the spotted phages. The Direct Spot test was also performed in triplicate (for a total of 27 plates). For each 3-plate set, one plate was incubated at 25°C, one at 28°C, and one at 30°C. After 24 hours, the plates were examined for prominent plaque formation within the boxed sections in each Spot assay plate. Five different bacteriophage samples from the plant sample enrichment culture were selected from the Spot assay plates that were incubated at 28°C. These phages demonstrated the formation of the clearest (least turbid) and largest plaques (based on diameter in mm) when compared to all other phage filtrate spots. To collect the selected phage samples, a sterile inoculation loop was used to touch the respective lytic plaque in its Spot assay grid box, and then subsequently to swirl the loop into a 1.5mL sterile Eppendorf tube containing 100μL of SM Buffer to get the phage cells in suspension. This is a buffer solution containing sodium and magnesium ions, which is commonly used in the long-term storage of bacteriophages [16]. After sample collection, the five phages were then ready for purification (isolation of lytic plaques to ensure a single viral species is present for each.

Purification of bacteriophage samples – Streak Plate Purification

Purification of our selected bacteriophage samples was meant to ensure the isolation of a single species from the mixed-species phage filtrates spotted in the previous test – known as a pure phage lysate. To obtain a pure lysate for each phage sample, a Streak Plate method of phage purification was performed 2-3 times, or until defined, isolated single lytic plaques could be observed for each of the five phage samples [35]. For each phage purification, a sterile inoculation loop was swirled in the previously prepared phage suspensions from the Direct Spot test, respectively, and then used to streak one third of a Nutrient Broth agar plate (stored in a 4°C refrigerator prior to phage streaking for quicker solidification of top agar overlay). Sterile inoculation loops were subsequently used to drag the initial inoculated streaked region onto the second and third quadrants of each plate. One purification plate was used for each phage sample (5 plates per round of phage purification). Once each phage sample was streaked onto its respective purification plate, a top agar solution was prepared for overlay onto each plate. The top agar recipe for each streak plate phage purification consists of the following: 4.5mL nutrient agar (nutrient broth+0.5% agar), and 500µL overnight *X. euvesicatoria* liquid culture (added after top agar cooled to ~48°C following autoclaving at 121°C, 15 psig, for 15 minutes). After preparing the 5mL combined top agar solution, it was then dispensed onto the most dilute region of the phage streak and gently overlayed, without swirling, onto each Nutrient Broth agar purification plate. After the top agar is allowed to solidify, each purification plate was incubated at 28°C for 48 hours. Following incubation of purification plates, phage stocks were prepared for each sample using the previously described method of suspension in SM Buffer. These steps were repeated for each round of phage purification. After the second round of purification, each of the five phage samples demonstrated the formation of isolated lytic plaques, which were then used to prepare the final purification stocks of each phage sample. Once each of the five phage

samples were purified, they were assigned a unique identifier based on the last names of each researcher who contributed to their isolation (**F**ernandez, **F**oerster, & **C**ollamore = FFC-1–5). This served as a temporary means of nomenclature for our bacteriophages, as they have not yet been identified or morphologically characterized using Transmission Electron Microscopy (TEM). For our previous study, the five purified phage samples were utilized for subsequent testing of calcium ion supplementation on phage lytic activity against *X. euvesicatoria* by performing a plaque assay (using the same approach described in the current study), and then subsequently stored in a 4°C refrigerator. However, for the current study, a re-evaluation of the plaque-forming abilities of our stored phage samples was necessary. Therefore, another 2-fold Streak Plate Purification was performed using the same protocol described above. After about a year of storage, only three of the five previously purified phage samples were still able to form lytic plaques. As a result, only Phages FFC-3, FFC-8, and FFC-11 were selected for subsequent plaque assay experimentation to test the lytic activity effects of sodium, potassium, magnesium, and zinc ion supplementation against *X. euvesicatoria* in the current study.

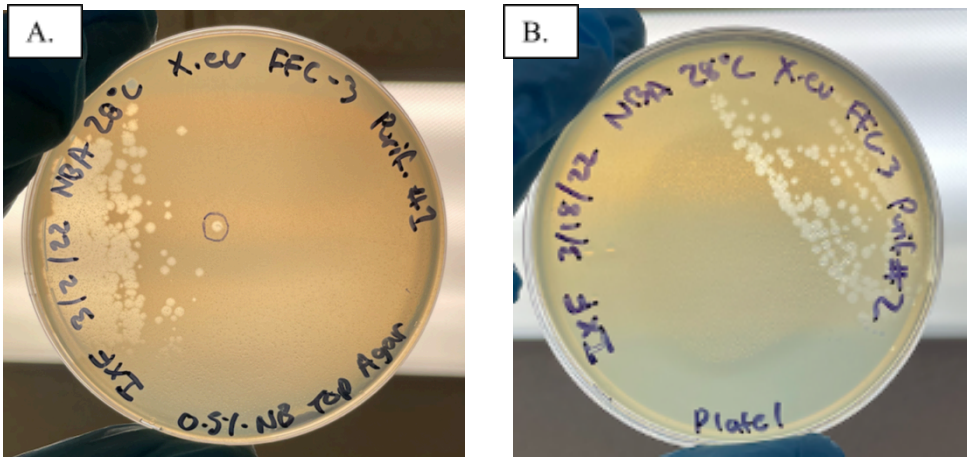

Figure 5: 2-fold streak plate purification results for Phage FFC-3. First round of purification for Phage FFC-3 (A.). Second round of purification for Phage FFC-3 (B.). Plaques appear more isolated and defined after the second of phage purification.

Determination of incubation time for a Xanthomonas euvesicatoria log phase culture – Growth Curve.

A key difference to consider between the streak plate phage purification protocol and the plaque assay protocol implemented in this study, is the quality of the *X. euvesicatoria* liquid culture used. When purifying a bacteriophage sample, the aim is to form isolated plaques, so as long as a full bacterial confluent lawn is able to grow, it should not interfere with the quality of those plaques. For this reason, an *X. euvesicatoria* liquid culture grown overnight was used phage purification. However, a plaque assay requires the use *X. euvesicatoria* cells in their logarithmic phase of growth prior to incubation to achieve the most qualitative plaques. The only information available in the literature regarding the growth phase characteristics of *X. euvesicatoria*, was that the start of its logarithmic phase was marked by an Optical Density (OD_{600}) value of 0.3 when measured using a spectrophotometer set at 600nm [12]. Therefore, a growth curve experiment was conducted to determine the amount of time required for a fresh culture of *X. euvesicatoria* cells to reach the start of the logarithmic phase for plaque assay experimentation [19]. To initiate this experiment, an overnight culture of *X. euvesicatoria* was prepared in a sterile test tube containing 10mL of 1.3% Nutrient Broth (NB). After 24 hours of incubation, the 10mL overnight culture was examined for signs of turbidity (indicator of bacterial growth in liquid culture) and subsequently added to a sterile 250mL Erlenmeyer flask containing 50mL of 1.3% Nutrient Broth. This was how the 60mL fresh culture was initially inoculated.

Prior to measuring the OD_{600} of the fresh culture, 1mL of 1.3% Nutrient Broth was transferred into a sterile cuvette and used to blank a spectrophotometer set to 600nm. A waste beaker was used to decant the contents of the cuvette after each use (same cuvette was used for

each OD measurement as a control; exterior of cuvette wiped with Kim wipe after each use). After decanting the blank, 1mL aliquots of the *X. euvesicatoria* overnight culture were transferred into the flask containing the 50mL of NB and measured in the spectrophotometer after every addition, until an adequate starting OD was reached (all spectrophotometer measurements involved transferring 1mL of the analyte into the cuvette and positioning it appropriately within the instrument). This was done to ensure that the first time point in the growth curve experiment (Time Point 0) had an OD_{600} reading between the range of 0.08–0.1 (an ideal starting OD for generating a bacterial growth curve). A starting OD of 0.1 was achieved after the addition of the entire 10mL contents of the overnight culture into the flask containing 50mL of NB. After measuring the OD for the first time point and between OD measurements for subsequent time points, the fresh culture flask was placed in a 28°C shaking incubator at 180rpm. The first two time points after Time Point 0 (Time Points 2 and 3), were spaced 30 minutes apart. Since the increases in OD at these increments were so small, subsequent time points were spaced 45 minutes apart. The final two time points (Time Points 7 and 8) were spaced 15 minutes apart. The *X. euvesicatoria* fresh culture achieved an OD_{600} value of 0.314 after exactly 5 hours (300 minutes) of incubation in a 28°C shaking incubator at 180rpm. This experiment was only performed once, as the findings shown in Table 1 determined that it takes 5 hours of incubation to prepare a log phase *X. euvesicatoria* fresh culture when inoculated with 10mL of overnight culture. This information was applied during subsequent plaque assay experimentation.

Time Point	Time (EST)	OD_{600}
0	4:11 PM (0 min.)	0.100
1	4:41 PM (30 min.)	0.108
2	5:11 PM (60 min.)	0.121
3	5:56 PM (105 min.)	0.150
4	6:41 PM (150 min.)	0.189
5	7:26 PM (195 min.)	0.230
6	8:01 PM (240 min.)	0.260
7	8:46 PM (285 min.)	0.295
8	9:01 PM (300 min.)	0.314

Table 1: Optical density measurements of a fresh *X. euvesicatoria* liquid culture at 9 different time points. The table above highlights the amount of time in min. allotted across each time point at which a spectrophotometer measurement was taken. When starting at an OD_{600} of 0.100, the measurements in this table indicate that it takes exactly 5 hours from the initial time point for the cells in the fresh culture to initiate the logarithmic phase of growth. Since the growth of *X. euvesicatoria* was not observed while undergoing log phase, the generation time of the bacterium could not be determined.

Cation supplementation of purified bacteriophage samples – Plaque Assay

To test the effect of various monovalent and divalent cations on the lytic activity of the three re-purified phage samples against *X. euvesicatoria*, a Plaque Assay was performed for each cation being tested [4,28]. Before initiating plaque assay experimentation, the following 10mM cationic salt master stock solutions were prepared using 500mL of DI H_2O: $CaCl_2$, $MgCl_2$, $ZnCl_2$, KCl, and NaCl. After master stock preparation, a 5-fold 1:10 serial dilution was performed for each solution by initially transferring 10mL of 10mM stock solution into a 250mL flask containing 90mL of DI H2O (to dilute 10mM to 1mM), and then repeating the same steps for

subsequent dilutions (to prepare 0.1mM, 0.01mM, and 0.001mM) until 5 flasks containing 90-100mL of the appropriate diluted solution are prepared and autoclave-sterilized for each cationic salt solution being tested. Following cationic salt solution dilution preparations, a 5-fold, 1:10 serial dilution was performed on each of the three previously re-purified phage sample stock solutions. In a similar manner to the dilutions of the cationic salt master stock solutions, 10µL of each phage stock was transferred into a sterile 1.5mL Eppendorf tube containing 90µL of SM Buffer for a 10^{-1} dilution. These steps were repeated until subsequent dilutions (10^{-2}–10^{-5}) of each phage were prepared.

It should be noted that the final three phage dilutions were used for plating to increase the probability of visible plaque formation after incubation. Since each Plaque Assay will be testing cationic salt supplementation of each phage sample at 5 different concentration levels (as well as a positive control condition of 0mM; phages without cation supplementation), a total of 54 plates will be utilized per Plaque Assay experiment (3 phage samples x 3 dilution factors plates x 6 cationic concentration levels = 54 total plates). Prior to the start of each Plaque Assay: a log phase *X. euvesicatoria* liquid culture was inoculated (10mL of overnight *X. euvesicatoria* liquid culture added to 50mL of Nutrient Broth media); 54 test tubes/caps were autoclave-sterilized at 121°C, 15 psig for 15 minutes; 54 Nutrient Broth agar plates were separated into 6 sets of 9 (according to concentration level) and placed in a 37°C incubator for 24–48 hours to ensure sterility and absence of contaminants; Nutrient Broth and agar reagents were weighed out for subsequent preparation of 360mL 0.5% top agar solution (DI H_2O not added until the time of the experiment) in a 750mL flask; and 6 250mL flasks were autoclave sterilized at 121°C, 15 psig for 15 minutes.

Each Plaque Assay experiment begins with the re-constitution of the 0.5% top agar mixture by adding 360mL of DI H_2O to the 750mL flask containing the pre-weighed reagents and dissolving/dispersing the solutes on a hot plate (with heat and magnetic stirring turned on). After top agar solution homogenizes, it is then placed into the autoclave for sterilization. While top agar is autoclaving, 100μL aliquots of log phase *X. euvesicatoria* liquid culture were dispensed into each of the 54 previously autoclave-sterilized test tubes (only after fresh bacterial culture has an OD_{600} of at least ~0.3). Once the top agar was done sterilizing in the autoclave, 60mL aliquots were dispensed into each of the 6 previously sterilized 250mL flasks. Each flask was labeled according to the cation concentration level being tested (0mM–10mM). For the five flasks labeled 0.001mM–10mM, 6.67mL of the appropriate cationic salt solution dilution were added to each, respectively, prior to adding the 0.5% top agar solution. This volume was calculated to mirror the ratio of the top agar recipe per test tube (used for overlay of each plate) when working with 60mL of total volume per flask. This recipe consists of the following: 4.5mL nutrient broth agar (nutrient broth+0.5% agar), and 0.5mL cationic salt solution at respective millimolar concentration per tube.

After each of the 6 top agar flasks are prepared, they were placed in a 48°C hot water bath to maintain the top agar at a workable temperature (hot enough that it stays as a liquid, but cool enough so that it will not kill the bacteria upon contact), while preventing its premature solidification. Prior to adding the 5mL aliquots of respective top agar solutions into respective test tubes, 10μL aliquots of the respective phage dilutions were added to each test tube containing the 100μL of *X. euvesicatoria* log phase culture and lightly vortexed. Once the phages are added to the bacteria, they were allowed to sit in suspension for 15-20 minutes to allow the phages time to attach to their host cells.

Following this stage of phage attachment, 5mL of the respective top agar solution were added to the respective test tubes containing 110μL of bacteriophage/bacteria suspension (addition of phages spaced 10 minutes apart; addition of top agar to bacteriophage/bacteria suspension spaced 15 minutes apart; addition of top agar performed one column (one flask condition; 15 plates) at a time to allow time for overlay. Once the 5.11mL mixtures were prepared in each of the respective test tubes, they were lightly vortexed, and subsequently poured/overlayed onto the respective Nutrient Broth agar plates (all 54 plates laid out to mirror the arrangement of test tubes and data table prior to the start of the experiment). Once all 54 plates have been overlayed with the top agar solution and allowed to solidify, they were re-taped into 6 sets of 9, and placed in a 28°C incubator for 48 hours. After incubation, plates were examined for observable plaque formation and analysis [27]. If quantifiable plaques formed, a Viral Titer calculation was then done for that plate. Viral Titer can be defined as the concentration of virulent phages in the stock solution, which is a value expressed in Plaque-forming Units per milliliter of solution (PFU/mL). The following formula was used to calculate Viral Titer: [# of plaques / (amount plated in mL x dilution factor)] = Viral Titer (PFU/mL). The above steps were repeated for each of the four Plaque Assay experiment performed in this study.

Results

Plaque assay plate observations for Phages FFC-3, FFC-8, and FFC-11 in the presence and absence of cation supplementation

To test the effect of $ZnCl_2$, $MgCl_2$, KCl, and NaCl, at five different concentrations, on bacteriophage activity against *Xanthomonas euvesicatoria*, a 5-fold, 1:10 serial dilution was performed on each of the three purified phage samples followed by a Plaque Assay. Tables 1–4 (one table for each cationic salt tested) depict the observable plaque assay results that were

recorded for each testing condition shown below. Only the last three dilution factors for each phage sample were plated (10^{-3}, 10^{-4}, & 10^{-5}). This was done due to the assumption that the first two dilutions would be too concentrated to demonstrate the formation of isolated and quantifiable plaques. For each incubated plate, the number of observable/quantifiable lytic plaques (clear circular zones absent of bacterial growth on the confluent lawn), the diameter of the largest observable plaque in mm, and the degree of turbidity (cloudiness) for the plaques that formed, were recorded. An ordinal and subjective turbidity scale ranging from 1–5 was created to represent the different levels of observable plaque cloudiness (1 = most turbid, 5 = least turbid). Only plaques with a turbidity level of 5 are clear enough to be considered lytic. Aside from the five cation concentrations that were tested, a positive control condition was also implemented in each of the four plaque assay experiments conducted in this study. Virulence activity of the three phage samples were tested against *X. euvesicatoria* in the absence of cation supplementation for the positive control. This was done to gauge the plaque-forming ability of each phage sample without addition supplementation, and to also establish a baseline for drawing comparisons between the induced effects of each cation on phages. The positive control was represented by a sixth cation concentration level labeled "0mM," in the second column of Tables 2–5. Plaque assay plates that yielded too many plaques to count were denoted by "TMTC." A quadrant method of quantification was used to determine the number of plaques present on plates that formed partially quantifiable plaques. This method involved counting the plaques on one quadrant of the plate (with the most isolated plaques to facilitate quantification) and then multiplying by 4 to obtain a plaque count approximation.

Phage sample / Dilution factor:	0mM:	0.001mM:	0.01mM:	0.1mM:	1.0mM:	10.0mM:
FFC-3, 10^{-3}	No plaques	No plaques	No plaques	No plaques	No plaques	No plaques
FFC-3, 10^{-4}	No plaques	No plaques	No plaques	No plaques	No plaques	No plaques
FFC-3, 10^{-5}	No plaques	No plaques	No plaques	No plaques	No plaques	No plaques
FFC-8, 10^{-3}	No plaques	No plaques	No plaques	No plaques	No plaques	No plaques
FFC-8, 10^{-4}	No plaques	No plaques	No plaques	No plaques	No plaques	No plaques
FFC-8, 10^{-5}	No plaques	No plaques	No plaques	No plaques	No plaques	No plaques
FFC-11, 10^{-3}	No plaques	No plaques	No plaques	No plaques	No plaques	No plaques
FFC-11, 10^{-4}	No plaques	No plaques	No plaques	No plaques	No plaques	No plaques
FFC-11, 10^{-5}	No plaques	No plaques	**Plaque count: 1 Plaque diameter (mm): 6mm Plaque turbidity: 4**	No plaques	No plaques	No plaques

Table 2: Plaque assay observations and measurements for KCl supplementation of Phages FFC-3, FFC-8, and FFC-11. After a 48-hour period of incubation at 28°C, the 54 plaque assay plates were examined for the presence of plaques on the confluent lawn of *X. euvesicatoria*. Dilution sets for each phage sample supplemented with 0mM, 0.001mM, 0.01mM, 0.1mM, 1.0mM, and 10mM of KCl were plated respectively. The number of quantifiable plaques, the diameter of the largest plaque present in mm, and the plaque turbidity score were recorded for each plate that formed observable plaques.

Phage sample / Dilution factor:	0mM:	0.001mM:	0.01mM:	0.1mM:	1.0mM:	10.0mM:
FFC-3, 10^{-3}	No plaques	No plaques	No plaques	No plaques	No plaques	Plaque count: 1 Plaque diameter (mm): 2.5mm Plaque turbidity: 2
FFC-3, 10^{-4}	No plaques	No plaques	No plaques	No plaques	No plaques	No plaques
FFC-3, 10^{-5}	No plaques	No plaques	No plaques	No plaques	No plaques	No plaques
FFC-8, 10^{-3}	No plaques	No plaques	No plaques	No plaques	No plaques	No plaques
FFC-8, 10^{-4}	No plaques	No plaques	Plaque count: 1 Plaque diameter (mm): 2.0mm Plaque turbidity: 4	No plaques	No plaques	No plaques
FFC-8, 10^{-5}	No plaques	No plaques	No plaques	No plaques	No plaques	No plaques
FFC-11, 10^{-3}	No plaques	No plaques	No plaques	No plaques	No plaques	No plaques
FFC-11, 10^{-4}	No plaques	No plaques	No plaques	No plaques	No plaques	No plaques
FFC-11, 10^{-5}	No plaques	No plaques	**Plaque count: 1 Plaque diameter (mm): 1.0mm Plaque turbidity: 4**	No plaques	No plaques	No plaques

Table 3: Plaque assay observations and measurements for NaCl supplementation of Phages FFC-3, FFC-8, and FFC-11. After a 48-hour period of incubation at 28°C, the 54 plaque assay plates were examined for the presence of plaques on the confluent lawn of *X. euvesicatoria*. Dilution sets for each phage sample supplemented with 0mM, 0.001mM, 0.01mM, 0.1mM, 1.0mM, and 10mM of NaCl were plated respectively. The number of quantifiable plaques, the diameter of the largest plaque present in mm, and the plaque turbidity score were recorded for each plate that formed observable plaques.

Phage sample / Dilution factor:	0mM:	0.001mM:	0.01mM:	0.1mM:	1.0mM:	10.0mM:
FFC-3, 10^{-3}	No plaques	Plaque count: 3 Plaque diameter (mm): 3.0mm Plaque turbidity: 4	Plaque count: 4 Plaque diameter (mm): 3.0mm Plaque turbidity: 3	Plaque count: 4 Plaque diameter (mm): 2.5mm Plaque turbidity: 4	Plaque count: 3 Plaque diameter (mm): 2.0mm Plaque turbidity: 5	Plaque count: 4 Plaque diameter (mm): 2.5mm Plaque turbidity: 3
FFC-3, 10^{-4}	No plaques	Plaque count: 2 Plaque diameter (mm): 2.0mm Plaque turbidity: 4	Plaque count: 3 Plaque diameter (mm): 4.5mm Plaque turbidity: 5	Plaque count: 1 Plaque diameter (mm): 4.0mm Plaque turbidity: 5	No plaques	No plaques
FFC-3, 10^{-5}	No plaques	No plaques	No plaques	No plaques	No plaques	No plaques
FFC-8, 10^{-3}	No plaques	Plaque count: TMTC Plaque diameter (mm): 4.0mm Plaque turbidity: 5	Plaque count: TMTC Plaque diameter (mm): 5.0mm Plaque turbidity: 5	Plaque count: (54 X 4) = ~216 Plaque diameter (mm): 4.0mm Plaque turbidity: 4	Plaque count: (96 x 4) = ~384 Plaque diameter (mm): 4.5mm Plaque turbidity: 5	Plaque count: (32 x4) = ~128 Plaque diameter (mm): 3.5mm Plaque turbidity: 4
FFC-8, 10^{-4}	No plaques	Plaque count: 15 Plaque diameter (mm): 4.5mm Plaque turbidity: 5	Plaque count: 102 Plaque diameter (mm): 5.0mm Plaque turbidity: 5	Plaque count: 78 Plaque diameter (mm): 4.0mm Plaque turbidity: 5	Plaque count: 65 Plaque diameter (mm): 4.5mm Plaque turbidity: 5	Plaque count: 61 Plaque diameter (mm): 4.0mm Plaque turbidity: 5
FFC-8, 10^{-5}	No plaques	Plaque count: 10 Plaque diameter (mm): 4.0mm Plaque turbidity: 5	Plaque count: 9 Plaque diameter (mm): 3.5mm Plaque turbidity: 5	Plaque count: 7 Plaque diameter (mm): 2.5mm Plaque turbidity: 5	Plaque count: 5 Plaque diameter (mm): 3.0mm Plaque turbidity: 5	Plaque count: 4 Plaque diameter (mm): 3.5mm Plaque turbidity: 3
FFC-11, 10^{-3}	No plaques	Plaque count: 11 Plaque diameter (mm): 4.0mm Plaque turbidity: 5	**Plaque count: 7 Plaque diameter (mm): 4.0mm Plaque turbidity: 5**	Plaque count: 10 Plaque diameter (mm): 4.0mm Plaque turbidity: 4	Plaque count: 5 Plaque diameter (mm): 4.0mm Plaque turbidity: 5	Plaque count: 4 Plaque diameter (mm): 2.5mm Plaque turbidity: 5
FFC-11, 10^{-4}	No plaques	No plaques	No plaques	No plaques	No plaques	Plaque count: 1 Plaque diameter (mm): 2.0mm Plaque turbidity: 4
FFC-11, 10^{-5}	No plaques	No plaques	No plaques	No plaques	No plaques	No plaques

Table 4: Plaque assay observations and measurements for $ZnCl_2$ supplementation of Phages FFC-3, FFC-8, and FFC-11. After a 48-hour period of incubation at 28°C, the 54 plaque assay plates were examined for the presence of plaques on the confluent lawn of *X. euvesicatoria*. Dilution sets for each phage sample supplemented with 0mM, 0.001mM, 0.01mM, 0.1mM, 1.0mM, and 10mM of $ZnCl_2$ were plated respectively. The number of quantifiable plaques, the diameter of the largest plaque present in mm, and the plaque turbidity score were recorded for each plate that formed observable plaques.

Phage sample / Dilution factor:	0mM:	0.001mM:	0.01mM:	0.1mM:	1.0mM:	10.0mM:
FFC-3, 10^{-3}	Plaque count: 3 Plaque diameter (mm): 3.0mm Plaque turbidity: 3	No plaques	No plaques	Plaque count: 1 Plaque diameter (mm): 1.5mm Plaque turbidity: 4	Plaque count: 1 Plaque diameter (mm): 1.0mm Plaque turbidity: 5	Plaque count: 1 Plaque diameter (mm): 2.0mm Plaque turbidity: 5
FFC-3, 10^{-4}	Plaque count: 1 Plaque diameter (mm): 0.5mm Plaque turbidity: 4	Plaque count: 2 Plaque diameter (mm): 1.0mm Plaque turbidity: 3	No plaques	Plaque count: 1 Plaque diameter (mm): 3.0mm Plaque turbidity: 5	No plaques	No plaques
FFC-3, 10^{-5}	No plaques	No plaques	No plaques	No plaques	No plaques	Plaque count: 1 Plaque diameter (mm): 1.0mm Plaque turbidity: 4
FFC-8, 10^{-3}	Plaque count: (51 X 4) = ~204 Plaque diameter (mm): 5.5mm Plaque turbidity: 5	Plaque count: 60 Plaque diameter (mm): 5.0mm Plaque turbidity: 5	Plaque count: 68 Plaque diameter (mm): 4.0mm Plaque turbidity: 5	Plaque count: 105 Plaque diameter (mm): 4.5mm Plaque turbidity: 5	Plaque count: 116 Plaque diameter (mm): 4.0mm Plaque turbidity: 5	Plaque count: (46 X 4) = ~184 Plaque diameter (mm): 3.5mm Plaque turbidity: 4
FFC-8, 10^{-4}	Plaque count: 16 Plaque diameter (mm): 4.0mm Plaque turbidity: 5	Plaque count: 8 Plaque diameter (mm): 3.5mm Plaque turbidity: 5	Plaque count: 13 Plaque diameter (mm): 4.0mm Plaque turbidity: 5	Plaque count: 12 Plaque diameter (mm): 4.0mm Plaque turbidity: 5	Plaque count: 23 Plaque diameter (mm): 4.0mm Plaque turbidity: 5	Plaque count: 22 Plaque diameter (mm): 4.0mm Plaque turbidity: 5
FFC-8, 10^{-5}	No plaques	Plaque count: 2 Plaque diameter (mm): 3.5mm Plaque turbidity: 5	Plaque count: 1 Plaque diameter (mm): 4.0mm Plaque turbidity: 5	Plaque count: 2 Plaque diameter (mm): 5.0mm Plaque turbidity: 5	Plaque count: 3 Plaque diameter (mm): 4.0mm Plaque turbidity: 5	Plaque count: 2 Plaque diameter (mm): 1.5mm Plaque turbidity: 5
FFC-11, 10^{-3}	Plaque count: 6 Plaque diameter (mm): 4.0 mm	Plaque count: 1 Plaque diameter (mm): 1.0mm	**Plaque count: 5** **Plaque diameter (mm): 3.5mm**	No plaques	Plaque count: 2 Plaque diameter (mm): 3.0mm Plaque turbidity: 5	Plaque count: 3 Plaque diameter (mm): 3.0mm Plaque turbidity: 5

	Plaque turbidity: 5	Plaque turbidity: 4	**Plaque turbidity: 5**			
FFC-11, 10^{-4}	No plaques	No plaques	No plaques	No plaques	Plaque count: 1 Plaque diameter (mm): 1.0mm Plaque turbidity: 3	No plaques
FFC-11, 10^{-5}	No plaques	No plaques	No plaques	No plaques	No plaques	No plaques

Table 5: Plaque assay observations and measurements for $MgCl_2$ supplementation of Phages FFC-3, FFC-8, and FFC-11. After a 48-hour period of incubation at 28°C, the 54 plaque assay plates were examined for the presence of plaques on the confluent lawn of *X. euvesicatoria*. Dilution sets for each phage sample supplemented with 0mM, 0.001mM, 0.01mM, 0.1mM, 1.0mM, and 10mM of $MgCl_2$ were plated respectively. The number of quantifiable plaques, the diameter of the largest plaque present in mm, and the plaque turbidity score were recorded for each plate that formed observable plaques.

Visualization of Phage FFC-11 plaque formation when supplemented with KCl, NaCl, $ZnCl_2$, and $MgCl_2$

Based on result evaluations for each plaque assay performed, it should be noted that a considerable degree of variance in plaque formation was observed among the four cations tested. Figure 4 shows a visualization of the plate results for Phage FFC-11, as it was the only phage sample that yielded observable plaques under the same supplementation condition for each of the four plaque assays performed. When supplemented with each cation at 0.01mM, Phage FFC-11 was able to form at least one observable plaque on the *X. euvesicatoria* confluent lawn. Each plate in Figure 4 yielded plaques that varied in count, diameter size, and turbidity score. A visualization of these plates is necessary for comparing the differences in the plaque(s) that formed after supplementing one of our phage samples with each cation at a lower concentration level (≤ 0.1mM).

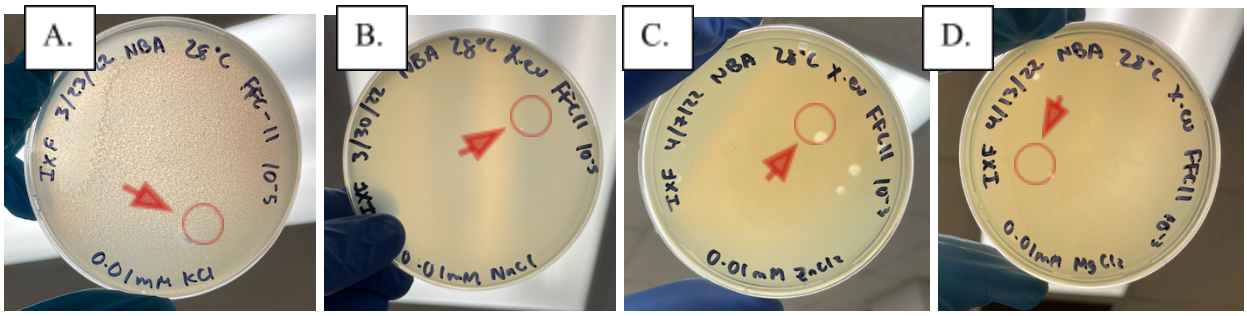

Figure 6: Visualization of plaque assay results for Phage FFC-11 after supplementation with each cation tested, at 0.01mM. The four images above depict the plaque assay results for Phage FFC-11 for the 0.01mM cation concentration condition. Phage FFC-11 yielded: 1 plaque of low turbidity with a diameter size of 6.0mm after supplementation with KCl (A.), 1 plaque of low turbidity with a diameter size of 1.0mm after supplementation with NaCl (B.), 7 lytic plaques with a diameter size of 4.0mm, for the largest plaque present, after supplementation with $ZnCl_2$ (C.), and 5 lytic plaques with a diameter size of 3.5mm, for the largest plaque present, after supplementation with $MgCl_2$ (D.).

Viral Titer of Phages FFC-3, FFC-8, and FFC-11 derived from plaque assay measurements

Following the tabulation of plaque assay measurements, a Viral Titer calculation was then performed for each individual plaque assay plate that demonstrated the formation of one or more observable plaques, per the previously recorded observations. The Viral Titer is a measure of the virions (infectious/lytic bacteriophage cells) concentration in each purified phage stock. This calculation was done using the formula: [# of lytic plaques / (amount of phage plated in mL x dilution factor)] = the Viral Titer in plaque-forming units per milliliter (PFU/mL). This calculated value served as a characteristic for drawing comparisons between the cations tested (focusing on the cationic atomic mass and valence), and the concentration levels of each cation used for supplementation, to highlight any prominent virulence effects on Phages FFC-3, FFC-8, and FFC-11. In Tables 6–9 (one table for each plaque assay), the calculated Viral Titer values in plaque-forming units per milliliter of solution (PFU/mL) are depicted for each phage sample that formed plaques when tested at each of the six cation concentration levels. In the case of plaque formation observed on more than one dilution factor for the same phage sample at a particular cation concentration level, an average Viral Titer was calculated and recorded in Tables 6–9,

respectively. Plaque assay plates that did not yield any plaque formation were not assigned a viral titer value. Furthermore, plaque assay plates that formed too many plaques to count (TMTC) were not factored in when calculating Viral Titer averages.

Phage sample:	KCl concentration (mM):	Viral Titer (PFU/mL):
FFC-3	0mM	No plaques
FFC-3	0.001mM	No plaques
FFC-3	0.01mM	No plaques
FFC-3	0.1mM	No plaques
FFC-3	1.0mM	No plaques
FFC-3	10mM	No plaques
FFC-8	0mM	No plaques
FFC-8	0.001mM	No plaques
FFC-8	0.01mM	No plaques
FFC-8	0.1mM	No plaques
FFC-8	1.0mM	No plaques
FFC-8	10mM	No plaques
FFC-11	0mM	No plaques
FFC-11	0.001mM	No plaques
FFC-11	**0.01mM**	**~1x10^{7} PFU/mL**
FFC-11	0.1mM	No plaques
FFC-11	1.0mM	No plaques
FFC-11	10mM	No plaques

Table 6: Calculated Viral Titer values for each phage sample tested at six cation concentration levels of KCl supplementation. The concentration of lytic phage cells in each purified phage stock was calculated using the formula: [# of plaques observed / (0.01mL x dilution factor)] = Viral Titer (PFU/mL). Viral Titer calculations were initially performed for each individual plaque-forming plate, and then averaged prior to tabulation if necessary. The plaque diameter and plaque turbidity score exhibited do not impact the Viral Titer, as it is a value dependent on the number of quantifiable plaques observed and the dilution factors of 10^{-3}–10^{-5}.

Phage sample:	NaCl concentration (mM):	Viral Titer (PFU/mL):
FFC-3	0mM	No plaques
FFC-3	0.001mM	No plaques
FFC-3	0.01mM	No plaques
FFC-3	0.1mM	No plaques
FFC-3	1.0mM	No plaques
FFC-3	**10mM**	**~1x10^{5} PFU/mL**
FFC-8	0mM	No plaques
FFC-8	0.001mM	No plaques
FFC-8	**0.01mM**	**~1x10^{6} PFU/mL**
FFC-8	0.1mM	No plaques
FFC-8	1.0mM	No plaques
FFC-8	10mM	No plaques
FFC-11	0mM	No plaques
FFC-11	0.001mM	No plaques
FFC-11	**0.01mM**	**~1x10^{7} PFU/mL**
FFC-11	0.1mM	No plaques
FFC-11	1.0mM	No plaques
FFC-11	10mM	No plaques

Table 7: Calculated Viral Titer values for each phage sample tested at six cation concentration levels of NaCl supplementation. The concentration of lytic phage cells in each purified phage stock was calculated using the formula: [# of plaques observed / (0.01mL x dilution factor)] = Viral Titer (PFU/mL). Viral Titer calculations were initially performed for each individual plaque-forming plate, and then averaged prior to tabulation if necessary. The plaque diameter and plaque turbidity score exhibited do not impact the Viral Titer, as it is a value dependent on the number of quantifiable plaques observed and the dilution factors of 10^{-3}–10^{-5}.

Phage sample:	$ZnCl_2$ concentration (mM):	Viral Titer (PFU/mL):
FFC-3	0mM	No plaques
FFC-3	**0.001mM**	**~$1.15x10^6$ PFU/mL**
FFC-3	**0.01mM**	**~$1.7x10^6$ PFU/mL**
FFC-3	**0.1mM**	**~$7x10^5$ PFU/mL**
FFC-3	**1.0mM**	**~$3x10^5$ PFU/mL**
FFC-3	**10mM**	**~$4x10^5$ PFU/mL**
FFC-8	0mM	No plaques
FFC-8	**0.001mM**	**~$9.75x10^7$ PFU/mL**
FFC-8	**0.01mM**	**~$9.6x10^7$ PFU/mL**
FFC-8	**0.1mM**	**~$5.65x10^7$ PFU/mL**
FFC-8	**1.0mM**	**~$5.11x10^7$ PFU/mL**
FFC-8	**10mM**	**~$3.79x10^7$ PFU/mL**
FFC-11	0mM	No plaques
FFC-11	**0.001mM**	**~$1.1x10^6$ PFU/mL**
FFC-11	**0.01mM**	**~$7x10^5$ PFU/mL**
FFC-11	**0.1mM**	**~$1x10^6$ PFU/mL**
FFC-11	**1.0mM**	**~$5x10^5$ PFU/mL**
FFC-11	**10mM**	**~$7x10^5$ PFU/mL**

Table 8: Calculated Viral Titer values for each phage sample tested at six cation concentration levels of $ZnCl_2$ supplementation. The concentration of lytic phage cells in each purified phage stock was calculated using the formula: [# of plaques observed / (0.01mL x dilution factor)] = Viral Titer (PFU/mL). Viral Titer calculations were initially performed for each individual plaque-forming plate, and then averaged prior to tabulation if necessary. The plaque diameter and plaque turbidity score exhibited do not impact the Viral Titer, as it is a value dependent on the number of quantifiable plaques observed and the dilution factors of 10^{-3}–10^{-5}.

Phage sample:	$MgCl_2$ concentration (mM):	Viral Titer (PFU/mL):
FFC-3	**0mM**	**~ $6.5x10^5$ PFU/mL**
FFC-3	**0.001mM**	**~$2x10^6$ PFU/mL**
FFC-3	0.01mM	No plaques
FFC-3	**0.1mM**	**~$5.5x10^5$ PFU/mL**
FFC-3	**1.0mM**	**~$1x10^5$ PFU/mL**
FFC-3	**10mM**	**~$5.05x10^6$ PFU/mL**
FFC-8	**0mM**	**~$1.82x10^7$ PFU/mL**
FFC-8	**0.001mM**	**~$1.13x10^7$ PFU/mL**
FFC-8	**0.01mM**	**~$9.93x10^6$ PFU/mL**
FFC-8	**0.1mM**	**~$1.42x10^7$ PFU/mL**
FFC-8	**1.0mM**	**~$2.15x10^7$ PFU/mL**
FFC-8	**10mM**	**~$2.01x10^7$ PFU/mL**
FFC-11	**0mM**	**~ $6x10^5$ PFU/mL**
FFC-11	**0.001mM**	**~$1x10^5$ PFU/mL**
FFC-11	**0.01mM**	**~$5x10^5$ PFU/mL**
FFC-11	0.1mM	No plaques
FFC-11	**1.0mM**	**~$6x10^5$ PFU/mL**
FFC-11	**10mM**	**~$3x10^5$ PFU/mL**

Table 9: Calculated Viral Titer values for each phage sample tested at six cation concentration levels of $MgCl_2$ supplementation. The concentration of lytic phage cells in each purified phage stock was calculated using the formula: [# of plaques observed / (0.01mL x dilution factor)] = Viral Titer (PFU/mL). Viral Titer calculations were initially performed for each individual plaque-forming plate, and then averaged prior to tabulation if necessary. The plaque diameter and plaque turbidity score exhibited do not impact the Viral Titer, as it is a value dependent on the number of quantifiable plaques observed and the dilution factors of 10^{-3}–10^{-5}.

Visualization of negative control and plaque turbidity scale

In addition to the experimental conditions being implemented in this study, as well as the positive control, we also plated a negative control when performing the Plaque assay experiment. Unlike the positive control condition, the negative control was only done one time, so no

tabulations were necessary for this condition. During the five-fold, 1:10 serial dilution step of the methodology, which was performed on each purified phage stock prior to plaque assay experimentation, we prepared a 10^{-1} dilution of DI H_2O in SM Buffer, which was then combined with the log phase *X. euvesicatoria* liquid culture and the top agar, for overlay plating. Figure 5 shows the plaque assay results of the negative control plate after the incubation period.

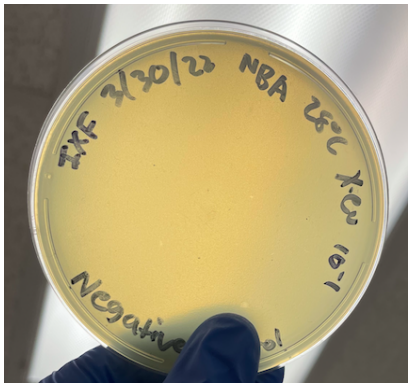

Figure 7: **Plaque assay plate result for the negative control.** This plaque assay plate demonstrates the growth of a *X. euvesicatoria* confluent lawn. Only one negative control was plated as an example to highlight how *X. euvesicatoria* grows on a plaque assay plate in the absence of cation supplementation and phage. DI H_2O was utilized in the serial dilution in place of a purified phage stock. This would equate to a plaque turbidity score of 0, which indicates an absence of plaques.

One of the parameters being observed in each plaque assay and recorded in Tables 2–5, is plaque turbidity. Plaque turbidity is a measure of how 'cloudy,' the plaques on a plate appear. According to the literature, a plaque can only be considered lytic when it is completely clear or lacks cloudiness entirely (turbidity score of 5). For the purposes of this study, the ideal result would be the formation of these clear lytic plaques because they serve as a strong indication of lytic activity against the host bacterium, and the presence of infectious phage cells (whether they are naturally lytic phages or temperate phages that have transitioned into the lytic cycle). Lytic plaques are also a characteristic of interest because one of the overarching goals of this study is to optimize the mechanism and improve the rate by which bacteriophages exhibit virulence

activity against a phytopathogen host (*X. euvesicatoria*) to achieve an increased degree of Bacterial Spot treatment effectiveness compared to copper-mancozeb bactericides. Plaque turbidity is an important characteristic to consider when interpreting plaque assay results because it allows the experimenter to gauge the lytic quality of the plaques that formed. These differences in plaque quality can also be compared across conditions to draw conclusions or implications regarding cation-induced virulence effects. Figure 11 depicts a visualization of five different plaque-forming phage purification plates. Each plate contains plaques that represent a score from 1–5 per the plaque turbidity scale (from left to right). A plaque turbidity score of 1 denotes the cloudiest that a plaque can appear before it is unable to be observed, and a plaque turbidity score of 5 denotes the clearest possible plaques that are completely absent of cloudiness.

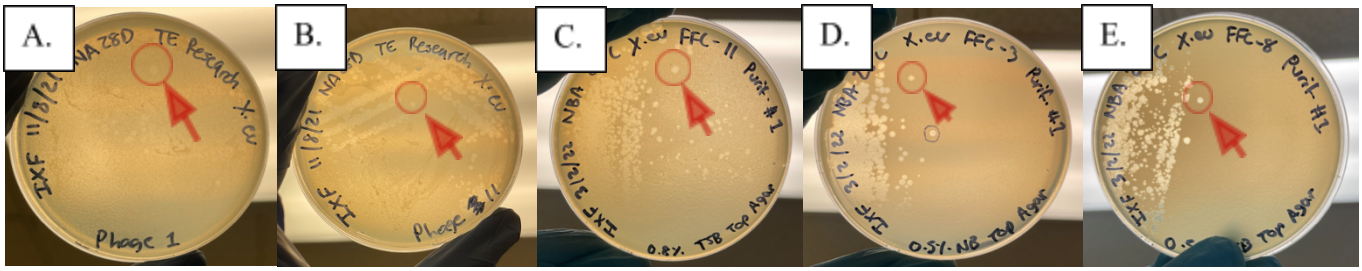

Figure 8: **Five different levels of decreasing plaque turbidity.** These images depict five different phage purification plates that each represent a level of plaque turbidity (cloudiness), as purported by the following subjective and ordinal turbidity scale: 1 = High turbidity (A.), 2 = Moderate turbidity (B.), 3 = Mild turbidity (C.), 4 = Low turbidity (D.), and 5 = No turbidity (E.). Although it is assumed that plaques are present if assigned a turbidity level from 1–5, only plaques that are completely clear (E.) can be considered lytic.

Due to the lack of a larger sample size of plaque-forming plates for each combination of plaque assay experimental conditions, a statistical analysis of the data presented in this study was unable to be conducted.

Discussion

Every plaque assay performed in this study tested the effect of cation supplementation on Phages FFC-3, FFC-8, and FFC-11 against *Xanthomonas euvesicatoria* – a previously isolated Bacterial Spot causal agent. Each cation implemented in this study was tested at the following six concentration levels for each phage sample: 0mM, 0.001mM, 0.01mM, 0.1mM, and 10mM. Each plaque assay consisted of 54 plates (3 dilution plates per condition), and 18 different conditions, with each condition being the combination of a particular phage sample with a particular cation concentration level. All plaque assays yielded the formation of plaques for at least one condition, which supported our first hypothesis that monovalent and divalent cation supplementation of our purified phage samples will have an observable effect on virulence activity (Tables 2–9). The first plaque assay, which tested KCl phage supplementation, yielded plaque formation for 1 condition (1 plate), which is the least amount of virulence activity observed from the four plaque assays performed (Tables 2 & 6). The second plaque assay, which tested NaCl phage supplementation, yielded plaque formation for 3 conditions (3 plates), (Tables 3 & 7). The third plaque assay, which tested $ZnCl_2$ phage supplementation, yielded plaque formation for 15 conditions (29 plates), (Tables 4 & 8). The fourth plaque assay, which tested $MgCl_2$ phage supplementation, yielded plaque formation for 16 conditions (31 plates), which is the most amount of virulence activity observed from the four plaque assays performed (Tables 5 & 9). Out of the three phage samples tested, only Phage FFC-11 formed observable plaques under the same cation concentration condition (0.01mM), in each of the four plaque assays (Figure 6). Furthermore, Phage FFC-8 demonstrated the most virulence activity against *X. euvesicatoria* , and yielded the highest average Viral Titer values of the other purified phage samples (15/29 plaque-forming plates for FFC-8 supplemented with $ZnCl_2$, and 17/31 plaque-forming plates for FFC-8 supplemented with $MgCl_2$), (Tables 2–9).

Out of the four cations tested in this study, Both, Zn^{2+} and Mg^{2+}, demonstrated plaque formation in significantly more plaque assay conditions than K^{+} and Na^{+}. Since the presence of clear, large, and quantifiable plaques are indicators of phage virulence, these findings suggest that divalent cations are more effective than monovalent cations in inducing virulence activity when used to supplement our purified phage samples against *X. euvesicatoria*. These implications parallel those presented in a previous study conducted on the human pathogen, *Bacillus cereus*, which tested the effects of divalent and monovalent cations on bacterial growth over time. The *B. cereus*-specific phages, BCP1-1 and BCP8-2 were able to effectively eradicate *B. cereus* from the fermented food system, but only if divalent cations were added to the medium [5]. This study also highlighted that divalent cations, such as Ca^{2+}, Mg^{2+}, or Mn^{2+} are essential for phage adsorption (adhesion to bacterial cell wall surface), while monovalent cations, such as Na^{+}, are required for the post-adsorption phase (DNA injection, lysis, and stabilization of viral particles) of phage infection [5]. However, cations such as Ca^{2+}, were selected for this particular study because of its known importance in the lytic life cycle of *B. subtilis* (a different bacterial species but belonging to the same genus as *B. cereus*) phages [5]. Therefore, the selected cations in the current study should be investigated further in order to determine whether they play a role in the lytic life cycle of *X. euvesicatoria* bacteriophages, and to progress on the development of a molecular pathway for defining these mechanisms.

Upon conducting a comparative analysis of the four plaque assays conducted in this study, it was deduced that more significant virulence activity was mostly exhibited by our purified phage samples when supplemented with cations at concentrations ≤ 0.1mM, which supported our second hypothesis. This deduction was based on the following plaque-forming plates that occurred at concentrations ≤ 0.1mM: 1/1 plaque-forming plates for KCl phage

supplementation, 2/3 plaque-forming plates for NaCl phage supplementation, 18/29 plaque-forming plates for $ZnCl_2$ phage supplementation, and 19/31 plaque-forming plates for $MgCl_2$ phage supplementation. In a previous adsorption study on the *Bacillus subtilis*-specific bacteriophage, Phage 41C, Ca^{2+} ion supplementation was tested on the degree of phage adsorption to the bacterial host [27]. This study showed that Phage 41C adsorption was efficient when supplemented with Ca^{2+} at 0.1mM, 1.0mM, and 10mM, but successful infection was only observed in the presence of Ca^{2+} 10mM [27]. Interestingly, the findings of this study suggested that low concentration of Ca^{2+} can promote adsorption of *B. subtilis* phages, but high concentrations are also necessary for a stage in infection beyond adsorption [27]. Further investigation is necessary to determine whether divalent and/or monovalent cations increase virulence activity against a *Xanthomonas* phytopathogen when used to supplement phytopathogen-specific bacteriophages at both high and low concentrations.

Another key finding obtained in the current study is that the fourth plaque assay, which tested Mg^{2+} supplementation, resulted in the formation of plaques under the most conditions (16) out of the four cations tested. Furthermore, this was the only plaque assay that yielded plaque formation for the control condition (0mM), so calculated Viral Titers can be compared to a baseline for determining any increases or decreases in virulence activity in our phage samples following supplementation with Mg^{2+} ion. This finding highlights the importance of Mg^{2+} and suggests that this cation may play a significant role in the lytic life cycle of *Xanthomonas* bacteriophages. Based on the Viral Titer results for the fourth plaque assay shown in Table 9, the concentration of lytic phages increased at 0.001mM and 10mM for Phage FFC-3, and at 1.0mM and 10mM for Phage FFC-8, but only stayed the same at 1.0mM for Phage FFC-11, when compared to the 0mM control condition. Moving forward, Mg^{2+} ions will remain as an ingredient

of consideration for the development of a more environmentally safe bacteriophage biocontrol formulation. However, since Mg^{2+} levels ≥ 8.5mM could be considered toxic for plant growth and development when implemented in a soil solution[15], further investigations with Mg^{2+} phage supplementation at lower concentrations is necessary to evaluate its continued implementation in our approach.

The plaque assay results of the current study contributed to the overarching significance of this research, which is to progress in the investigation of alternative methods for improving the virulence activity of bacteriophages against a phytopathogen host, in an effort to develop a more effective Bacterial Spot disease control agent than copper-mancozeb. Further investigation is necessary to determine whether $ZnCl_2$ (being the heaviest of the cations tested and adjacent to Cu^{2+} on the periodic table) poses any immediate non-target consequences for the environment. This is important because if heavy ions, such as Zn^{2+}, have the potential to cause negative non-target environmental impacts, then they should be excluded from further consideration in the development of our proposed phage biocontrol formulation.

Based on the findings presented, future studies could consider a multitude of directions moving forward. Due to the promising phage virulence activity effects induced by $ZnCl_2$ and $MgCl_2$, the same plaque assay approach used in the current comparative study can be modified to test the virulence effect induced by supplementation of our purified phage samples with combinations of two or more divalent and/or monovalent cations (i.e., $MgCl_2 + ZnCl_2$; $MgCl_2 + ZnCl_2 + CaCl_2$; $ZnCl_2 + NaCl$, etc.) at concentrations ≤ 0.1mM. Another upcoming direction could be an *in vivo* study on a healthy tomato plant grown under controlled laboratory conditions to investigate the treatment potential of cation-supplemented bacteriophages in combatting the pathogenicity of *X. euvesicatoria* over time, and to investigate any negative non-target

environmental impacts that may be caused by the cations used. Lastly, an analytical study of our three plaque-forming purified bacteriophage samples could be conducted for morphological characterization, proper identification, and appropriate assignment of nomenclature, using Transmission Electron Microscopy (TEM).

References

1. *AgriPhage-CMM Organic Ingredients List*. Agriphage-CMM. (n.d.). Retrieved April 17, 2022, from https://www.plantproducts.com/m/ca/viewproduct.php?pid=507
2. *Application of agricultural silicone oil penetrant.* Jiande City Silibase Import and Export Co.,Ltd. (n.d.). Retrieved April 17, 2022, from https://www.silibasesilicone.com/info/application-of-agricultural-silicone-oil-penet-50950501.html
3. Arahamian, P., Jones, J. B., & Vallad, G. E. (2019). Efficacy of copper and copper alternatives for management of bacterial spot on tomato under transplant and field production. *Crop Protection*, ***126***, 104919. doi:https://doi.org/10.1016/j.cropro.2019.104919
4. Baer, A., & Kehn-Hall, K. (2014). Viral concentration determination through plaque assays: using traditional and novel overlay systems. *J. Vis. Exp.*, ***93***, e52065. doi:https://doi.org/10.3791/52065
5. Bandara, N., Jo, J., Ryu, S., & Kim, K. (2012). Bacteriophages BCP1-1 and BCP8-2 require divalent cations for efficient control of *Bacillus cereus* in fermented foods. *Food Microbiol.*, ***31***(1), 9–16. doi:https://doi.org/10.1016/j.fm.2012.02.003
6. Bascom, C. S., Hepler, P. K., & Bezanilla, M. (2018). Interplay between ions, the cytoskeleton, and cell wall properties during tip growth. *Plant Physiol., **176***, 28–40. doi:https://doi.org/10.1104/pp.17.01466
7. Bashan, Y., Azaizeh, M., Diab, S., Yunis, H., & Okon, Y. (1985). Crop loss of pepper plants artificially infected with *Xanthomonas campestris pv. vesicatoria* in relation to symptom expression. *Crop Protection, **4***, 77–84. doi:https://doi.org/10.1016/0261-2194(85)90007-9
8. Buttimer, C., McAuliffe, O., Ross, R. P., Hill, C., O'Mahony, J., & Coffey, A. (2017). Bacteriophages and Bacterial Plant Diseases. *Front. Microbiol*. ***8***, 34. doi:https://doi.org/10.3389/fmicb.2017.00034
9. Cabot C., Martos S., Llugany M., Gallego B., Tolrà R., & Poschenrieder C. (2019). A Role for Zinc in Plant Defense Against Pathogens and Herbivores. *Front. Plant Sci., **10***, 1171. doi:https://doi.org/10.3389/fpls.2019.01171
10. Conover, R. A., & Gerhold, N. R. (1981). Mixture of Copper and Maneb or Mancozeb for Control of Bacterial Spot of Tomato and their Compatibility for Control of Fungus Diseases. *Proc. Fla. State Hortic. Soc., **94***, 154–156.
11. Drulis-Kawa, Z., Majkowska-Skrobek, G., Maciejewska, B., Delattre, A. S., & Lavigne, R. (2012). Learning from bacteriophages - advantages and limitations of

phage and phage-encoded protein applications. *Curr. Protein Pept. Sci*., ***13***(8), 699–722. doi:https://doi.org/10.2174/138920312804871193

12. Gašić, K., Ivanović, M. M., Ignjatov, M., Calić, A., & Obradović, A. (2011). ISOLATION AND CHARACTERIZATION OF XANTHOMONAS EUVESICATORIA BACTERIOPHAGES. *Journal of Plant Pathology*, ***93***(2), 415–423. http://www.jstor.org/stable/41999013
13. Gašić, K., Kuzmanović, N., Ivanović, M., Prokić, A., Šević, M., & Obradović, A. (2018). Complete Genome of the Xanthomonas euvesicatoria Specific Bacteriophage KΦ1, Its Survival and Potential in Control of Pepper Bacterial Spot. *Front. Microbiol*., ***9***, 2021. doi:https://www.frontiersin.org/article/10.3389/fmicb.2018.02021
14. Gill, J. J., & Hyman, P. (2010). Phage choice, isolation, and preparation for phage therapy. *Curr. Pharm. Biotechnol., **11,*** 2–14. doi:https://doi.org/10.2174/138920110790725311
15. Guo, W., Chen, S., Hussain, N., Cong, Y., Liang, Z., & Chen, K. (2015). Magnesium stress signaling in plants: just a beginning. *Plant Signal Behav*., ***10***(3), e992287. doi:https://doi.org/10.4161/15592324.2014.992287
16. Gutiérrez, D., Fernández, L., Rodríguez, A., & García, P. (2018). Practical Method for Isolation of Phage Deletion Mutants. *Methods Protoc*., ***1***, 6. doi:https://doi.org/10.3390/mps1010006
17. Helfenstein, J., Pawlowski, M. L., Hill, C. B., Stewart, J., Lagos-Kutz, D., Bowen, C. R., Frossard, E., & Hartman, G. L. (2015). Zinc deficiency alters soybean susceptibility to pathogens and pests. *J. Plant Nutr. Soil Sci., **178***, 896–903. doi:https://doi.org/10.1002/jpln.201500146
18. Hermans, C., & Verbruggen, N. (2005). Physiological characterization of Mg deficiency in *Arabidopsis thaliana*. *J. Exp. Bot*., ***56***, 2153-2161. doi:https://doi.org/10.1093/jxb/eri215
19. Hernandez, S., et al. (2018). Tiny Earth: A Research Guide to Studentsourcing Antibiotic Discovery. *XanEdu Publishing*, Acton, MA. 50–52; 169–176; 185–189.
20. Iriarte, F. B., Obradović, A., Wernsing, M. H., Jackson, L. E., Balogh, B., Hong, J. A., Momol, M. T., Jones, J. B., & Vallad, G. E. (2012). Soil-based systemic delivery and phyllosphere in vivo propagation of bacteriophages: two possible strategies for improving bacteriophage persistence for plant disease control. *Bacteriophage*, ***2***, 215–224. doi:https://dx.doi.org/10.4161%2Fbact.23530
21. Jones, J. B., Jones, J. P., Stall, R. E., & Zitter, T. A. (1991). Compendium of Tomato Disease. *America Phytopathological Society Press,* St. Paul, 73.
22. Jones, J. B., & J. P. Jones. (1985). The efect of bactericides, tank mixing time and spray schedule on bacterial leaf spot of tomato. *Proc. Fla. State Hortic. Soc*. ***98***, 244–247.

23. Jones, J. B., Lacy, G. H., Bouzar, H., Stall, R. E., & Schaad, N. W. (2004). Reclassification of the Xanthomonads Associated with Bacterial Spot Disease of Tomato and Pepper. *Syst. Appl. Microbiol., **27***, 755–762. doi:https://doi.org/10.1078/0723202042369884
24. King, A. M. Q., Adams, M. J., Carstens, E. B., & Lefkowitz, E. J. (2012). Family Myoviridae. *Virus Taxonomy*. 46–62. doi:https://doi.org/10.1016/B978-0-12-384684-6.00002-1
25. Koller, W. (1998). "Chemical Approaches to Managing Plant Pathogens." In *Handbook of Integrated Pest Management*, edited by J. R. Ruberson. Dekker.
26. Kumar, J., & Doshi, A. (2018). Pathogenicity Test and Convenient Inoculation Method of *Xanthomonas axonopodis pv. vignaeradiatae* Caused Lead Spot of Green gram. *Int. J. Curr. Microbiol. App. Sci., **7***(4), 152-166. doi:https://doi.org/10.20546/ijcmas.2018.704.017
27. Landry, E. F. (1975). EFFECT OF CALCIUM ION ON THE LYTIC CYCLE OF BACTERIOPHAGE 41C. *Doctoral Dissertations*, 1085. https://scholars.unh.edu/dissertation/1085?utm_source=scholars.unh.edu%2Fdissertation%2F1085&utm_medium=PDF&utm_campaign=PDFCoverPages
28. Lee, A. (2020). Plaque Assay. *protocols.io*. doi:https://dx.doi.org/10.17504/protocols.io.be6sjhee
29. Maathuis, F. J. M. (2014). Sodium in plants: perception, signaling, and regulation of sodium fluxes. *J. Exp. Bot*., ***65***(3), 849–858. doi:https://doi.org/10.1093/jxb/ert326
30. Marco, G. M., & R. E. Stall. (1983). Control of Bacterial Spot of Pepper Initiated by Strains of *Xanthomonas campestris* pv. *vesicatoria* That Differ in Sensitivity to Copper. *Plant Dis., **67***, 779–781. doi:https://doi.org/10.1094/PD-67-779
31. McGuire, R. G., Jones, J. B., & Sasser, M. (1986). Tween Media for Semiselective Isolation of *Xanthomonas campestris pv. vesicatoria* from Soil and Plant Material. *Plant Dis*., ***70***, 887–891. doi:https://doi.org/10.1094/PD-70-887
32. Nazarov, P. A., Baleev, D. N., Ivanova, M. I., Sokolova, L. M., & Karakozova, M. V. (2020). Infectious Plant Diseases: Etiology, Current Status, Problems and Prospects in Plant Protection. *Acta naturae*, ***12***(3), 46–59. doi:https://doi.org/10.32607/actanaturae.11026
33. Obradovic, A., Jones, J. B., Momol, M. T., Olson, S. M., Jackson, L. E., Balogh, B., Guven, K., & Iriarte, F. B. (2005). Integration of Biological Control Agents and Systemic Acquired Resistance Inducers Against Bacterial Spot on Tomato. *Plant Disease, **89***(7), 712–716. doi:https://doi.org/10.1094/PD-89-0712
34. Osdaghi, E., Jones, J. B., Sharma, A., Goss, E. M., Abrahamian, P., Newberry, E. A., Potnis, N., Carvalho, R., Choudhary, M., Paret, M. L., Timilsina, S., & Vallad, G. E. (2021). A centenary for bacterial spot of tomato and pepper. *Mol. Plant Pathol*., ***22***(12), 1500–1519. doi:https://doi.org/10.1111/mpp.13125

35. Poxleitner, M., Pope, W., Jacobs-Sera, D., Sivanathan, V., & Hatfull, G. (2018). Phage Discovery Guide. *The Science Education Alliance – Phage Hunters Advancing Genomics and Evolutionary Science.* https://seaphagesphagediscoveryguide.helpdocsonline.com
36. Principi, N., Silvestri, E., & Esposito, S. (2019). Advantages and Limitations of Bacteriophages for the Treatment of Bacterial Infections. *Front. Pharmacol., **10***, 513. doi:https://doi.org/10.3389/fphar.2019.00513
37. *Product info*. AgriPhage. (n.d.). Retrieved April 17, 2022, from https://www.agriphage.com/product-info/
38. Ramesh, N., Archana, L., Madurantakam Royam, M., Manohar, P., & Eniyan, K. (2019). Effect of various bacteriological media on the plaque morphology of Staphylococcus and Vibrio phages. *Access Microbiology*, ***1***(4). doi:https://doi.org/10.1099/acmi.0.000036
39. Ray, D. K., Mueller, N. D., West, P. C., & Foley, J. A. (2013). Yield trends are insufficient to double global crop production by 2050. *PLoS ONE*, ***8***, e66428. doi:https://doi.org/10.1371/journal.pone.0066428
40. Ryan, R. P., Vorhölter, F. J., Potnis, N., Jones, J. B., Van Sluys, M. A., Bogdanove, A. J., & Dow, J. M. (2011). Pathogenomics of *Xanthomonas*: understanding bacterium–plant interactions. *Nat. Rev. Microbiol., **9***, 344–355. https://pubmed.ncbi.nlm.nih.gov/21478901/
41. *Safety Data Sheet - Sigmaaldrich.com*. (n.d.). Retrieved April 17, 2022, from https://www.sigmaaldrich.com/US/en/sds/SIAL/151173
42. Silipo, A., Molinaro, A., Sturiale, L., Dow, J. M., Erbs, G., Lanzetta, R., Newman, M., & Parrilli, M. (2005). The Elicitation of Plant Innate Immunity by Lipooligosaccharide of *Xanthomonas campestris*. *Glycobiology and Extracellular Matrices*, ***280***(39), P33660–33668. doi:https://doi.org/10.1074/jbc.M506254200
43. Strange, R. N., & Scott, P. R. (2005). Plant disease: a threat to global food security. *Annu. Rev. Phytopathol., **43***, 83–116. doi:https://doi.org/10.1146/annurev.phyto.43.113004.133839
44. Strayer-Scherer, A., Liao, Y., Abrahamian, P., Timilsina, S., Paret, M., Momol, T., Jones J., & Vallad, G. E. (2019). Integrated Management of Bacterial Spot on Tomato in Florida. *askifas powered by EDIS*, ***6***, 8. https://edis.ifas.ufl.edu/publication/PP353
45. Sulakvelidze, A., Alavidze, Z., & Morris, J. G. (2001). Bacteriophage therapy. *Antimicrob. Agents Chemother., **45***, 649–659. doi:https://doi.org/10.1128/AAC.45.3.649-659.2001
46. Thayer, P. L., & Stall, R. E. (1961). A Survey of *Xanthomonas vesicatoria* Resistance to Streptomycin. *Proc. Fla. State Hortic. Soc., **75***, 163–165.
47. Thor, K. (2019). Calcium–Nutrient and Messenger. *Front. Plant Sci*., ***10***, 440. doi:https://doi.org/10.3389/fpls.2019.00440

48. Wang, M., Zheng, Q., Shen, Q., & Guo, S. (2013). The critical role of potassium in plant stress response. *Int. J. Mol. Sci*., ***14***(4), 7370–7390. doi:https://doi.org/10.3390/ijms14047370
49. Weinbauer, M. G. (2004). Ecology of prokaryotic viruses. *FEMS Microbiol. Rev., **28***, 127–181. doi:https://doi.org/10.1016/j.femsre.2003.08.001
50. Živković, S., Stojanović, S., Ivanović, Ž., Trkulja, N., Dolovac, N., Aleksić, G., & Balaž, J. (2010). *Pestic. Fitomed*., ***25***(3), 231–239. doi:https://doi.org/10.2298/PIF1003231Z