STRATEGIES FOR IMPROVING THE EFFICACY OF BACTERIOPHAGES FOR CONTROLLING BACTERIAL SPOT OF TOMATO

By

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Bacterial spot, caused by the bacterium *Xanthomonas campestris* pv. *vesicatoria*, is one of the major tomato diseases in Florida. The disease is routinely controlled by the application of copper-mancozeb, a mixture of chemical pesticides; however, there is no adequate control measure when the environmental conditions are conducive for disease development. A novel method for controlling this disease is the application of a mixture of bacteriophages, viruses that infect bacteria. However, these control agents are rapidly degraded by harmful environmental factors such as sunlight or desiccation, which delimits the efficacy of phage treatment. It has been hypothesized that the efficacy of phage treatment could be enhanced if the longevity of the viruses was increased.

Three formulations were developed that enhanced the longevity of bacteriophages on plant foliage. These formulations were (i) PCF (0.5% pregelatinized corn flour (PCPF 400, Lauhoff Grain Co., Danville, IL) + 0.5% sucrose), (ii) Casecrete (0.5% Casecrete

NH-400, a water-soluble casein protein polymer (American Casein Company, Burlington, NJ)+ 0.5% sucrose + 0.25% PCPF 400)), and (iii) skim milk (0.75% powdered skim milk + 0.5% sucrose). The use of these formulations resulted in a 4,700, 38,500 and 100,000-fold increase in phage populations two days after application compared to the non-formulated phage populations.

The PCF, Casecrete and skim milk formulations and the non-formulated phages all significantly reduced disease severity in field trials on tomato compared to the standard copper-mancozeb treatment by 22, 33, 27 and 19%, respectively. The PCF and the Casecrete formulations reduced the disease severity compared to the non-formulated phage by 11 and 21% in average in three field experiments, respectively. The skim milk formulation reduced the disease severity by 10% compared to the non-formulated phage application in one field experiment.

The co-application of skim milk-formulated phages and copper-mancozeb treatment resulted in a superior disease control efficacy, which was significantly better than the control achieved by any of the treatments. The integration of phage application with Actigard treatment resulted in a significant increase in efficacy only with the PCF formulation but not with Casecrete formulation. The lowest phage titer that significantly reduced disease development in greenhouse experiment was 10^6 PFU/ml.

CHAPTER 1
INTRODUCTION

Fresh-market tomato (*Lycopersicon esculentum* Mill.) is the third most popular vegetable product in the United States. Florida agriculture is responsible for about half of the domestic fresh-market production, while it is basically the only provider of fresh-market tomatoes during the winter and spring seasons (Florida Tomato Committee, 2001). Fresh-market tomato production is a major component of Florida agriculture. In 1999-2000, 17,500 hectares were used for tomato production in Florida. The production value was $418,348,000, which ranked tomato as the number one vegetable crop in Florida, accounting for 27 percent of the total vegetable production value of the state (Florida Agricultural Statistics Service, 2001).

Tomatoes are subject to a number of diseases that result in economic losses to the producer. One of the most devastating diseases of tomato in Florida and the Caribbean is bacterial spot (Bouzar et al., 1999; Scott and Jones, 1984), which can cause up to 50 percent losses in fruit production and makes the fruit unacceptable for fresh market consumption (Pohronezny and Volin, 1983).

The causal agent of the bacterial spot disease, which was first observed in 1914 in South Africa (Doidge, 1920), is the gram-negative bacterium, *Xanthomonas campestris* pv. v*esicatoria* (Xcv). The bacterium is favored by high temperatures (25-30 °C) and a wet environment. Under optimal conditions the disease is highly contagious. The bacterium survives on diseased plant debris and volunteer plants (Jones et al., 1986), is disseminated by wind-driven rain (Volcani, 1969), and enters the plant through wounds

and natural openings, such as stomates and hydothodes (Vakili, 1967; Kucharek, 1994). The characteristic symptom of the disease is the development of small, 1-3 mm diameter, dark colored spots, with or without yellow halos, that occur on leaves, fruits and stem. Later the spots enlarge and coalesce with each other, leading to browning of the entire leaflet, and subsequently to defoliation (Kucharek, 1994).

X. campestris pv. *vesicatoria* causes the bacterial spot disease of pepper in addition to tomato and can be found virtually everywhere where tomato and pepper are grown (Stall, 1995). According to their pathogenicity profile, Xcv strains can be divided into three groups: the strains that are pathogenic only on tomato belong to the XcvT group; the ones pathogenic only on pepper are the members of the XcvP group; and the strains that cause disease on both tomato and pepper constitute the XcvTP group (Jones et al., 1998a). The tomato strains are divided into three races, according to their reaction on differential genotypes: H7998, H7981 and Bonny Best (Jones et al., 1998a).

According to genetic and phenotypic characteristics, the Xcv strains were divided into two major groups, A and B, which were later reclassified as *X. axonopodis* pv. *vesicatoria* and *X. vesicatoria*, respectively (Vauterin et al., 1995). There have been some problems relating to reclassification of the A group strains to *X. axonopodis*, since Jones et al. (2000) determined that A and C strains had less than 60% homology with *X. axonopodis*. Since it is the responsibility of the scientific society to accept or reject changes in classification, I will continue to use previous nomenclature of *X. campestris* pv. *vesicatoria*. Tomato race 1 (T1), into which all Florida tomato strains were shown to fall originally, belongs to group A, while tomato race 2 (T2), which is widely distributed throughout the world but has not been found in Florida, belongs to group B. In 1991 a

new tomato race, T3, appeared in Florida (Jones at al., 1995), and was placed into a distinct third group, group C. It has been suggested that group C is actually a subgroup of A (Jones, 1998a). Interestingly, the T3 strains have a competitive advantage over the T1 strains in the greenhouse and field (Jones et al., 1998b). This advantage is a result of the production of at least three bacteriocin-like substances by T3 strains that specifically inhibit the growth of T1 strains (Tudor, 1999).

Management of this disease may be achieved by the integration of cultural practices, chemical control and resistance breeding. However, "during periods of wind-driven rains, no available control measures are adequate" (Kucharek, 1994).

Cultural practices focus mainly on disease prevention and provide the backbone for bacterial spot management. The destruction of crop residues and volunteer tomato and pepper plants, disinfection of seeds, and separation of transplant beds from pepper or tomato fields are the important means for prevention of the disease. Additionally, since the disease is spread by rain splash, it is advised to use drip irrigation instead of overhead irrigation (Kucharek, 1994).

Chemical control originally relied on application of streptomycin, an antibiotic, and copper compounds. However, streptomycin lost its effectiveness due to the emergence of resistant strains in the 1960s (Thayer and Stall, 1961) and by the 1980s copper resistant strains emerged as well (Marco and Stall, 1983). Eventually, the copper bactericides also became ineffective when used alone (Marco and Stall, 1983); however, it was discovered that the addition of maneb or mancozeb fungicides to the copper bactericides enhances their efficacy (Marco and Stall, 1983). Since then these copper-mancozeb mixtures have been in use for controlling bacterial spot, although complete

control cannot be achieved even with them. In the fall growing season, when the weather conditions favor disease development, growers may spray more than two times a week and still not achieve sufficient disease control.

Development of resistant varieties has resulted in effective suppression of a number of plant diseases and has been attempted in this pathosystem as well (Nelson, 1973; Birkenhoff et al. 1984). The tomato genotype Hawaii 7998 was determined to carry genetic resistance against T1 strains (Scott and Jones, 1986); however, the emergence of T3 strains (Jones et al., 1995), which have become prevalent in Florida (Jones et al., 1998b) and which overcome this resistance, has hindered the use of this resistance in Florida. Several tomato genotypes have been identified that are resistant to T3 strains (Jones et al., 1995), although Minsavage and Jones identified T3 strains that have overcome these sources of resistance (unpublished). Additionally, several T3 strains were shown to possess mobile genetic elements in their genome that contributed to higher mutation rate in the host's avirulence genes leading to emergence of virulent progeny (Balogh, Minsavage and Jones, unpublished).

Recently, alternative chemical control approaches have been investigated in which chemicals are applied that activate plant defense responses (Cartwright et al., 1977). Systemic acquired resistance (SAR) is a natural physiological state of plants that have previously been attacked by pathogens, which provides plant resistance to a wide range of pathogens (Sticher et al., 1997). Several substances that specifically induce SAR, such as acibenzolar-S-methyl and harpin, have shown activity against bacterial diseases of tomato (Louws et al., 2001; Qui et al., 1997) and their use in disease control is the subject of ongoing research (Obradovic et al., in press).

Biological control could be achieved using several approaches. One approach is to use antagonistic bacteria. T1 bacteria, which are inhibited by T3 strains in the greenhouse and field (Hert, 2001; Jones et al., 1998b; Tudor-Nelson et al., 1995), were successfully controlled with an avirulent strain of the T3 bacterium (Liu, 1998). In other studies, *Pseudomonas putida* B56 and *P. syringae* Cit7 reduced bacterial spot in field trials (Wilson et al., 1997). Plant growth promoting rhizobacteria (PGPR), that induce SAR, have been successfully used for controlling bacterial speck of tomato (Ji et al., 1996).

Other biological agents that have been used to control bacterial spot are bacterial viruses or bacteriophages (phages). Phages have long been proposed as plant disease control agents (Moore, 1926) and have been used in several plant-bacterium pathosystems. However, reliable disease control was not achieved with them in the past. Recently, Jackson (1989) proposed an approach that uses a mixture of host-range mutant bacteriophages, and in subsequent field trials twice-weekly spray application of the phage mixture reduced the disease severity of bacterial spot by 17 percent, whereas the copper-mancozeb application caused 11 percent reduction (Flaherty et al., 2000).

However, it was noted that the phage treatment was effective only if applied very early in the morning, before dawn, presumably because the virus particles perished very fast in the daytime. Therefore, the need arose to develop formulations and/or change the application strategy such as time of application in order to protect phage particles from harmful environmental factors; thus, the residual activity of the viruses would increase and could lead to increased efficacy of phage treatments and to a more convenient application schedule. The objective of this research project was to develop such

protective formulations and to identify other application strategies to increase the efficacy of bacteriophage treatments.

CHAPTER 2
LITERATURE REVIEW

The Use of Bacteriophages for Treatment and Prevention of Bacterial Diseases

More than a hundred years ago, in 1896, Hankin discovered that the water of Ganges and Jumma rivers in India possessed bactericidal activity on *Vibrio cholerae* (Sharp, 2001). This activity remained after filtering but disappeared upon boiling. He speculated that drinking river water would be effective against the spread of cholera, but did not examine the phenomenon further.

The discovery of bacteriophages came about two decades later, when Twort (1915) and d'Herelle (1917) independently reported about filterable and transmissible agents of bacterial lysis. They did not agree, though, on the origin of this "lytic principle". While Tworth proposed that a bacterial enzyme caused the lysis, d'Herelle speculated that a virus was responsible for the phenomenon. Even though d'Herelle's bacterial virus theory had not been proven conclusively for over 30 more years, the idea of using phages as control agents of bacterial diseases was a direct consequence of his concept.

Great enthusiasm captured the medical society with the possible use of phages as a tool for fighting many bacterial diseases that did not have any effective control measures. Shortly after d'Herelle's discovery, Brunoghe and Maisin (1921) already reported the control of *Bacillus anthracis* and *Staphylococcus* infections with bacteriophage treatment; a year later phages were evaluated for the control of typhoid

fever and bacillary dysentery (Beckerich and Hauduroy, 1922; Davison, 1922). Phages were also used extensively to control cholera outbreaks in India (Morrison, 1932). Over 800 papers were published over the next 40 years concerning disease control or prevention with bacteriophages (Kutter, 1997).

Phages were evaluated for control of plant diseases as well. Mallman and Hemstreet (1924) isolated the "cabbage-rot organism" from rotting cabbage and demonstrated that the filtrate of the liquid obtained from the decomposed cabbage inhibited the growth of the pathogen *in vitro*. Kotila and Coons (1925) isolated bacteriophages from soil samples active against the causal agent of blackleg disease of potato, *Bacillus atrosepticus* (*Erwinia carotovora* subsp. *atroseptica*). They demonstrated in growth chamber experiments that co-inoculation of *Bacillus atrosepticus* with phage successfully inhibited the pathogen and prevented rotting of tubers (Kotila and Coons, 1925). These workers also isolated phages against *Bacillus carotovorus* (*Erwinia carotovora* subsp. *carotovora*) and *Bacterium tumefaciens* (*Agrobacterium tumefaciens*) from various sources, such as soil, rotting carrots and river water (Coons and Kotila, 1925). Thomas (1935) reported effective control of Stewart's wilt disease of corn due to treatment with bacteriophage isolated from diseased plant material against the disease-causing bacterium *Aplanobacter stewarti* (*Panthoea stewartii*). The average amount of disease decreased from 18 to 1.4 percent in plots where phage-treated seed were used, and the distribution of the disease was reduced as well.

In spite of this early enthusiasm the bacteriophages did not become the "silver bullet" for bacterial disease control; the phage therapy did not prove to be an effective control method due to lack of understanding of the biology behind the bacteriophage

phenomenon and as a result of poor experimental techniques (Kutter, 1997). Even where effective control was achieved with phages it was unreliable and in many cases successes were questionable due to largely uncontrolled experiments. Eventually, after the discovery of antibiotics that proved to be much more effective bactericides, the use of bacteriophages as biological control agents was largely abandoned and most of the phage research groups changed their focus to basic research.

Bacteriophages became the tool of molecular biology and genetics. Significant discoveries were made by examining the phage-bacterium interaction, such as that nucleic acid is the genetic material (Hershey and Chase, 1952); that the ability of bacteria to produce phages, lysogeny, is inherited (Lwoff, 1953), and the bacteriophage-sensitive bacteria is capable of becoming bacteriophage-resistant via spontaneous mutation (Luria and Delbrück, 1943). Additionally, the phases of phage infection, from adsorption to the burst of the host cell, and physiologic background were characterized. Later it became clear that two types of bacteriophages exist: *lytic* phages that always infect from the outside with their infection resulting in the destructive burst of the host cell and release of phage progeny, and *temperate* phages that can insert their DNA into the host's DNA and stay in association with the host cell in an inactive form, as a prophage, without destroying it. The term lysogenic bacterium refers to the phenomenon in which prophages produce a repressor that provides resistance for the host against related and sometimes also unrelated phages. Prophages sometimes carry genes that function as virulence factors for the host bacterium or carry bacterial genes to a new bacterial host in a process called transduction (Adams, 1959; Birge, 2000).

As a result of the increased knowledge of bacteriophages some authors warned against using bacteriophages as control agents. Vidaver (1976) listed five reasons why phages are not recommended for plant disease control:

1. Virulent bacteriophages can become temperate and the lysogenized bacteria would become resistant to the phage as well as to related ones.
2. The bacteriophages could transduce virulence factors from one host to another; possibly even enable originally non-pathogenic organisms to become pathogenic.
3. "Transducing phages can introduce active prokaryotic genes into plant and animal cells" producing genetically modified organisms (GMO) *in situ*.
4. Environmental conditions could alter bacteriophage pathogenicity, including the restriction of host-range.
5. Resistant bacterial strains can emerge due to mutation.

However, bacteriophages still remained in use in plant pathology: they became tools for classification and identification of pathogenic bacteria by a method called *phage typing*. Due to the highly specific virus-host recognition, phage typing could differentiate between closely related pathogenic bacterial groups, such as different races or pathovars of the same bacterial species (Klement et al., 1990).

Recent events such as the appearance of bacterial strains that are resistant to all known antibiotics as well as the lack of discovering new antibiotics resulted in renewed interest in phage therapy in the field of medicine. The greater knowledge and advanced research techniques led to successful treatment of several human bacterial diseases in Poland (Slopek et al., 1987) and to the development and commercial application of a wide range of phage-based medical products in the former Soviet republics of Georgia and Russia, such as sprays, ampoules, and pills for treatment of various skin and enteric diseases. In these systems there was a great emphasis on building a bank of pathogenic

bacterial strains and phages that were capable of lysing them (Kutter, 1997). Initially, the host range, lytic spectrum and cross-resistance properties of the phage strains were evaluated. Only lytic phages were used for therapy avoiding the problems caused by lysogeny. In order to prevent the problem of resistance, a phage cocktail was prepared from strains with different receptor specificity for the pathogenic bacterium, sometimes for several different species of pathogenic bacteria (Kutter, 1997). For example, a formulation called IntestiPhage, developed by the Eliava Institute in Georgia, contained 23 different phages active against a wide range of enteric bacteria (Kutter, 1997). Phage formulations were successfully used not only for disease treatment but also for prevention (Solodovnikov et al., 1970) as well as for sanitation purposes (Kutter, 1997).

There are several potential advantages for using phages in disease control:

1. Bacteriophages are natural components of the biosphere; they can be readily isolated from everywhere where bacteria are found, including soil, water, plants, animals (Adams, 1959) and the human body (Osawa et al., 1981). Phages can be used in situations when chemical control is not allowed due to legal regulations such as in organic production.
2. Phages are self-replicating and self-limiting, because they replicate only as long as the host bacterium is present in the environment, but are quickly degraded when the host is absent (Kutter 1997).
3. Bacteriophages are non-toxic. Daily, humans consume bacteriophages, which are found in raw produce and fermented products without getting harmed (Jackson, 1996). Additionally, no serious side effects have been reported in phage therapy

trials even though phage particles have been detected in many organs and tissues, such as the bloodstream and brain (Barrow 2001).

4. They are specific, eliminating only target bacteria without damaging other members of the environment. This also means that phage application can be coupled with the introduction of other biological control agents such as bacteria that are competitors or antagonists of the pathogen. Some positive results have been obtained with such coupled treatment in medicine (Litvinova et al., 1978) as well as in plant pathology (Tanaka et al., 1990).
5. Bacteriophages may be targeted to bacterial receptors that are virulence factors, so the bacteria that become resistant to the phages would possess reduced pathogenic ability. Antibody fragments that are specific to bacterial antigens have been expressed in phage as fusion proteins, enabling the specific and planned targeting of phage to the desired surface receptor on the bacterial host and thus effective control of the pathogen (Cao et al., 2000).
6. Phage preparations are fairly inexpensive to produce. No specific equipment is needed, thus every laboratory capable of growing bacteria could produce phages. Additionally, the main cost factor of phage production is labor and the price of culture media ingredients (Jackson, 1996).
7. The preparations can be stored at 4 °C for years without significant reduction in titer (Jackson, 1996).
8. The constituent phage strains can be altered in the mixture to adapt the cocktail to possibly emerging new bacterial strains.

As a result of the expanding knowledge base about phage application in medicine, the appearance of copper resistant bacterial strains in the field (Marco and Stall, 1983), and the need for environment-friendly pesticides, there was a renewed interest in developing bacteriophage-based disease control methods in modern agriculture. Saccardi et al. (1993) reduced the fruit spot incidence on peaches with biweekly spray applications of phage suspension effective against *Xanthomonas campestris* pv. *pruni*, the causal agent of the disease. They started by isolating eight phages active against the pathogen, screening them for host range and lytic ability, and selecting a lytic phage strain with the broadest host range for disease control (Zaccardelli et al., 1992; Saccardi et al., 1993).

Tanaka et al. (1990) successfully controlled tobacco bacterial wilt, caused by *Ralstonia solanacearum*, by co-application of an avirulent *R. solanacearum* strain and its bacteriophage that was active against the virulent strains of the pathogen as well. They reduced the ratio of wilted plants from 95.8 percent of control, to 39.5 and 17.6 percent with the application of avirulent strain and avirulent strain plus phage, respectively.

Control of *Erwinia amylovora*, the fire blight pathogen of apple, pear and raspberry, with bacteriophages is currently under investigation in Canada and the USA. Gill et al. (1999) isolated forty-seven phages in Canada capable of lysing *E. amylovora* and categorized them based on plaque morphology and host range. Further characterization of the phages based on restriction enzyme digestion profile and PCR analysis, and the assessment of disease control potential of the phages is the subject of current research (Gill et al., 1999). Very similar research with the same goals is under way in the US (Schnabel and Jones, 2001).

Jackson (1989) developed a novel system for phage application; he prepared a mixture of host range mutant (h-mutant) bacteriophages for disease control. H-mutants are phages that possess the ability to lyse bacterial strains that are resistant to the parent phage (Adams, 1959), while still possessing the ability to lyse the wild type bacterium. Thus, they have an extended host range compared to the parent phage. Using a mixture of four h-mutant phages, Jackson (1989) was able to completely eliminate *Pseudomonas syringae* from contaminated bean cull. Bacterial spot disease of tomato was effectively controlled in greenhouse and field experiments with a mixture containing four h-mutant phages effective against the two predominant tomato races of Xcv (Flaherty et al., 2000).

A similar strategy was used to control *Xanthomonas campestris* pv. p*elargonii*, (Xcp) the causal agent of bacterial blight of geranium (Flaherty et al., 2001). Sixteen phages were evaluated for the ability to lyse Xcp strains isolated from around the world and then h-mutants were developed from five phages that exhibited the broadest host range and included in the mixture that was used for disease control. Foliar applications of the phage mixture applied daily significantly reduced the spread of disease. The disease incidence was reduced by 50 percent or more in phage-treated plots compared to the control, and was significantly less than in plots treated with the recommended bactericide.

The use of bacteriophages for disease control is a quickly expanding area of plant protection with great potential to replace the recently widespread chemical control measures in a number of plant diseases. Phages can be used effectively as part of integrated disease management strategies. The relative ease of preparing phage treatments and the low cost of production of these agents makes them a good candidate

for widespread use in developing countries as well. However, the efficacy of phages, as is true of all biological control agents, depends greatly on the surrounding environmental factors, as well as on the susceptibility of the target bacterium. A great deal of care is necessary during development, production and application of phage treatments. In addition, constant monitoring for the emergence of resistant bacterial strains is essential. It is important to understand that the phage-based disease control management is a dynamic process with a need for continuous adjustment of the phage preparation in order to effectively fight potentially adapting pathogenic bacteria.

Development of Protective Formulations for Microbial Pesticides

The large-scale use of pesticides is an essential component of modern agricultural practices. Chemicals are generally cheaper, faster acting and more convenient than other technologies, such as mechanical and cultural controls (Reichelderfer and Barry, 1995). Moreover, the risk factor of farming, especially in horticultural production, is high, thus pesticides are used to reduce variability. However, some widely used chemicals turned out to be harmful to the environment and even to humans. Pesticides are under increased review by the U. S. Environmental Protection Agency (EPA) and increased scrutiny by the press. Concerns focus on pesticides in food, air and water and the resulting impact on man and other organisms. The EPA's Safer Pesticide Policy and goals for new pesticides include: safer materials, less persistence, less toxicity to non-target species, less likely to contaminate ground water, lower exposure for man and environment, and more practical disposal technology (Reichelderfer and Barry, 1995).

These trends have led the industry toward the application of biorationals and biological control agents. Biocontrol, according to Merriam-Webster's Collegiate

Dictionary (1998), is the reduction in numbers or elimination of pest organisms by interference with their ecology. The essence of biological control is that the pest's natural antagonists are used for their control, such as parasites, predators or competitors of the target pest species. According to the summary of the 1995 American Chemical Society Symposium about the Biorational Pest Control Agents, viruses, bacteria and fungi have already been successfully applied against a wide range of pests (Reichelderfer and Barry, 1995).

One area where microbial control agents have been widely and successfully used is in insect control. The bacterium *Bacillus thuringiensis* (Bt) has proven to be very effective for controlling certain insect pest groups and has been commercially available for decades for the control of the orders Lepidoptera, Coleoptera and Diptera (McGuire and Shasha, 1995). Similarly, baculoviruses have been used worldwide for the control of several Lepidoptera species; for example the nuclear polyhedrosis virus of the soybean caterpillar (*Anticarsia gemmatalis*) is used on approximately one million hectares annually in Brazil (Moscardi, 1999).

However, it was discovered on both the Bt and baculovirus system that harmful environmental factors seriously reduced the residual activity of these microbial agents, hindering the feasibility of their use (McGuire and Shasha, 1995). For instance, the half-life for persistence of baculoviruses in the field was two to five days, while in some cases the viral activity was completely lost in 24 hours (Moscardi, 1999). These problems led to extended research on identification of the most harmful environmental factors that affect microbial pesticides in the field, and to the pursuit of identifying substances that could provide effective protection against these factors.

Field and laboratory studies have demonstrated that microbial pesticides are inactivated by exposure to high temperature, alkalinity, low pH, sunlight, and rain/dew (Ignoffo & Garcia, 1992). The most destructive environmental factor was determined to be the ultraviolet-A and ultraviolet-B spectrum (280-400 nm) of sunlight (Ignoffo and Garcia, 1994). Also, the presence of free water could significantly increase the extent of virus inactivation by sunlight (Ignoffo & Garcia, 1992). For example the half-life of several insect viruses was less than 3 h after direct exposure on glass to simulated sunlight-UV (Ignoffo et al., 1991).

Sunlight ultraviolet (SUV) inactivation of field applied viral pesticides has been attributed to its direct effects on DNA, such as generating deleterious cross-linkings, strand breaks, development of labile sites, as well as the generation of highly reactive radicals that would degrade the biological agent (Ignoffo & Garcia, 1994). Ignoffo & Garcia (1994) examined several antioxidants and oxidative enzymes to determine whether they can inhibit the UV inactivation, and found an antioxidant (n-propyl-gallate) and an oxidative enzyme (catalase), which provided relatively good UV protection at low concentration. The propyl-gallate provided 99% protection at 0.2 mg/ml, while the catalase gave 90% protection at 10 mg/ml when viral pesticides were exposed to artificial sunlight for 24 h.

Aromatic/heterocyclic amino acids may also provide UV protection, since they absorb within the biologically active sunlight-UV spectrum. Tryptophan provided significant protection at a low concentration (0.1 mg/ml) providing 95% protection in 24 h exposure to simulated sunlight-UV (Ignoffo & Garcia, 1995).

There have been a number of attempts to increase the stability of viral particles when exposed to sunlight by using different formulations. Several dyes including Benzopurpurin (Ignoffo et al., 1997), Congo red (Ignoffo et al., 1991), and the fluorescent whitening agent, Tinopal LPW (Tamez-Guerra et al., 2000b), were shown to be effective UV protectants.

Carbon (activated charcoal) gave significant SUV and rain leaching protection (Ignoffo et al., 1991, Ignoffo et al., 1997). More than 97% of the original viral activity of carbon formulations was still present after 10 h exposure to SUV, and less than 6% activity was lost after a drenching, simulated rainfall.

Microencapsulation technologies have also been used to enhance the solar stability and rain fastness of microbial pesticides. A number of experiments have been done with granulated and sprayable starch- and flour-based formulations (Ignoffo et al., 1991; McGuire et al., 1990; 1994; 1996; Tamez-Guerra et al., 2000a). The formulations were not only effective, but economically advantageous as well.

Alkaline gluten formulations improved the residual activity of microbial pesticides because the solubilized wheat-gluten forms a film upon drying that resist wash-off (Behle et al., 1997). A concentration of 1.5% (wt/v) gluten at pH 10.5 provided more than 80% wash-off protection after 50 mm simulated rain.

Casein-based formulations also provided wash-off resistance and some protection from light-induced degradation (Behle et al. 1996). Seventy one percent of the original activity of *Bacillus thuringiensis* remained with 0.5% (wt/v) casein-salt formulation after 50 mm simulated rainfall.

Lignin-based formulations provided good protection against both sun-UV and wash-off (Tamez-Guerra et al. 2000a). Lignin and corn flour formulations of *Bacillus thuringiensis* showed 35% more activity after simulated rain, and 12% more activity after simulated sunlight than Dipel 2X, a commercially used insecticide containing *Bacillus thuringiensis*. Additionally, the lignin and corn flour-based formulation had significantly higher residual activity than Dipel 2X, 4 days after application.

In summary, several substances have been reported to offer some level of protection against harmful environmental factors for microbial pesticides. The use of such protectants could significantly increase the time frame in which the biocontrol agent is present in a high enough population in the target area to be efficient for control, thus enabling the reduction of applied dose and the number of applications. This would then result in a more cost-effective treatment. In addition, the employment of protective formulations can allow utilization of novel, more fragile, biological agents under field conditions.

CHAPTER 3
DEVELOPING FORMULATIONS FOR IMPROVING PHAGE LONGEVITY ON LEAF SURFACES

Bacteriophages have been used for controlling bacterial spot on tomato (Flaherty et al., 2000). In that study twice weekly applications of a mixture of h-mutant bacteriophages contributed to the significant reduction of disease and significant increase in yield compared to the standard bactericide. However, the phage treatment had to be applied in the early morning, before dawn, in order to achieve proper control. It was anticipated that the phages were degraded rapidly during daytime and that they would not have a significant bactericidal effect if applied later in the day.

In order to increase the efficacy of phage treatment and to allow for a more convenient time for application, it would be necessary to increase the phage residual activity on the plant foliage. Increased longevity could additionally result in reduced application frequency and reduced dose.

A strategy for enhancing the residual activity of bacteriophage mixture is to use formulations that would protect the viruses from harmful environmental factors. Active research has been conducted on enhancing the longevity of microbial pesticides in the area of insect control, where viral and bacterial agents are used to fight several economically important insect pests. The main environmental factors contributing to the residual activity of microbial pesticides were identified as: sunlight ultra violet irradiation, rain wash-off, and exposure to high temperatures (Ignoffo & Garcia, 1992). In

addition, the majority of phages are also known to be sensitive to desiccation (Adams, 1959).

A number of agents and formulations have been identified, which were reported to prolong the residual activity of insect viruses and *Bacillus thuringiensis* on plant surfaces. A number of these are listed in Table 3-1.

The objective of this research was to investigate how long the commercially available *Xanthomonas campestris* pv. *vesicatoria* phage mixture remains active on the plant surface and to develop formulations, based on the reported materials (Table 3-1) that could effectively prolong phage activity on plant foliage.

Materials and methods

Bacterial culture and bacteriophage mixture. *Xanthomonas campestris* pv. *vesicatoria* strain BD2 (tomato race 3) was used for recovery and enumeration of bacteriophages. The bacterium was grown at 28°C on nutrient agar (0.8% (wt/V) nutrient broth (BBL, Becton Dickinson and Co., Cockeysville, MD) and 1.5% (wt/V) Bacto Agar (Difco, Becton Dickinson and Co., Sparks, MD)). The phage mixture contained six phages active against Xcv T3 bacteria (AgriPhi Inc., Logan, UT) and was stored at 4°C in dark until use.

Preparation of treatments. The formulations were prepared freshly before application and the phage mixture was added to each formulation at the end. The list of the formulations used in the experiments is provided in Table 3-2. The phage mixture was used at 10^8 PFU/ml. The formulations were delivered onto the foliage of tomato plants with hand-held sprayers.

Table 3-1. Materials reported to increase the longevity of microbial pesticides

Protective agent	Method of protection	Reference
N-propyl-gallate	Antioxidant- counteracts the damaging action of oxidation done by of reactive radicals generated by sunlight	Ignoffo and Garcia, 1994
Catalase	Oxidative enzyme – catalyses the degradation of reactive radicals generated by sunlight	Ignoffo and Garcia, 1994
Tryptophan	Absorb in the spectrum of sunlight UV A or B.	Ignoffo and Garcia, 1995
Benzopurpurin, Congo Red	Dyes that absorb in the spectrum of sunlight UV A or B.	Ignoffo et al., 1991 Ignoffo et al., 1997
Tinopal LPW	Fluorescent whitening agent that absorbs in the spectrum of sunlight UV A or B	Tamez-Guerra et al., 2000b
Activated charcoal	Sunlight and rain wash-off protection	Ignoffo et al., 1991
Starch- and flour-based formulations	Sunlight and rain wash-off protection	Ignoffo et al., 1991; McGuire et al., 1990; 1994; 1996; Tamez-Guerra et al., 2000a
Alkaline gluten formulations	Rain wash-off protection	Behle et al., 1997
Casein based formulations	Wash-off resistance and sunlight protection	Behle et al., 1996
Lignin-based formulations	Sunlight UV and wash-off protection	Tamez-Guerra et al., 2000a
Sucrose	Wash-off protection	McGuire and Shasha, 1995

Table 3-2. Formulations tested in this study for improving bacteriophage longevity on leaf surfaces

Name	Ingredients
	July 6, 2000 experiment
Non-formulated	Deionized water only
Gluten control	1% wheat gluten, (Sigma Chemical Co., St. Louis, MO) + 0.05% KOH (Sigma)
Congo Red	1% wheat gluten + 0.05% KOH + 0.1% Congo Red, Allied Chemical, Rochester, NJ
Tryptophan	1% wheat gluten + 0.05% KOH + 0.1 % Tryptophan (Sigma)
Propyl-gallate	1% wheat gluten + 0.05% KOH + 0.1 % n-propyl-gallate (Sigma)
Composite	1% wheat gluten + 0.05% KOH + 0.1% Congo Red+ 0.1% Tryptophan+ 0.1% n-propyl-gallate
	October 15 and 25, 2000 experiments
Lignate	0.5% Sodium lignate PC-1307 (Westvaco, Charleston Heights, SC) + 0.05% Calcium chloride dehydrate (Fisher Scientific Co., Fair Lawn, NJ)
PCF	0.5% PCF +0.5% sucrose
PCF #2	0.5% Pregelatinized corn flour (PCF) (PCPF 400, Lauhoff Grain Co., Danville, IL) +0.5% Sucrose (Mallinckrodt Inc. Paris, KY) + 0.005% Lactic acid 85% (Fisher) + 0.15% Mazola Corn Oil (Unilever Bestfoods) + 10% Isopropanol (Sigma)
Lignate+PCF	0.3% Na-lignate + 0.03% $CaCl_2$ + 0.2% PCF + 10% isopropanol
PCF+Gluten	0.25% wheat gluten + 0.05% KOH + 0.25% PCF
Non-formulated	Deionized water only
	November 15, 2000 experiment
Casein	0.5% Casein hydolysate (ICN Biochemicals Inc., Aurora, OH) + 0.05% KOH
Non-formulated	Deionized water only
PCF	0.5% PCF +0.5% sucrose
PCF+Silwet	0.5% PCF+ 0.5% sucrose + 0.1% Silwet L-77® (Loveland Industries Inc., Greely, CO)
PCF+Casein	0.25% PCF + 0.5% sucrose + 0.25 Casein hydolysate + 0.05% KOH
Silwet	0.1% Silwet L-77®
	March 22, 2001 experiment
Casecrete (0.5%)	0.5% Casecrete ™ NH-400 (American Casein Company, Burlington, NJ) + 0.5% sucrose+ 0.25% PCF
Casecrete (0.25%) + PCF (0.25%)	0.25% Casecrete + 0.25% PCF
BSA (0.1%)	0.1 % Bovine Serum Albumin (Sigma)
Gelatin (0.5%)	0.5 % Granular gelatin (Fisher)
Non-formulated	Deionized water only

Table 3-2-continued

Name	Ingredients
	March 28, 2001 experiment
Casecrete (0.5%) + sucrose (0.5%) + PCF (0.25%)	0.5% Casecrete + 0.25% PCF + 0.5% sucrose
Casecrete (0.5%) + sucrose (0.5%)	0.5% Casecrete + 0.5% sucrose
Casecrete (0.5%)+ PCF (0.25%)	0.25% Casecrete + 0.25% PCF
Casecrete (0.5%)	0.5% Casecrete
Non-formulated	Deionized water only
	March 28, 2001 experiment
Skim milk (0.5%) + gelatin (0.5%)	0.5% Dehydrated skim milk (Difco Laboratories, Detroit, MI) + 0.5% Granular gelatin
Skim milk (0.5%)	0.5% Dehydrated skim milk
Casein (0.5%)	0.5% Casein hydolysate + 0.05% KOH
Casecrete (0.5%)	0.5% Casecrete
Non-formulated	Deionized water only
	June 28, 2001 experiment
Casecrete (0.5%)+sucrose (0.5%) +PCF (0.25%)	0.5% Casecrete + 0.5% sucrose+ 0.25% PCF
Casecrete (0.75%) + sucrose (0.5%)	0.75% Casecrete + 0.5% sucrose
Casecrete (0.5%) + sucrose (0.5%)	0.5% Casecrete + 0.5% sucrose
Skim milk (0.5%) + sucrose (0.5%)	0.5% Dehydrated skim milk + 0.5% sucrose
Casein (0.5%) + sucrose (0.5%)	0.5% Casein hydolysate + 0.5% sucrose + 0.05% KOH
Casecrete (0.5%)	0.5% Casecrete
Skim milk (0.5%)	0.5% Dehydrated skim milk
Casein (0.5%)	0.5% Casein hydolysate + 0.05% KOH
PCF (0.5%) + sucrose (0.5%)	0.5% PCF + 0.5% sucrose
Miragel (0.5%) +sucrose (0.5%)	0.5% Mira-Gel 463 starch (A. E. Stanley Manufacturing Company, Decatur, IL) + 0.5% sucrose + 0.05% KOH
Finomliszt (0.5%) + sucrose (0.5%)	0.5% Finomliszt (Fine Wheat Flour, Hungary) + 0.5% sucrose + 0.05% KOH
All purpose flour (0.5%) + sucrose (0.5%)	0.5% All purpose flour (General Mills Sales Inc. Minneapolis, MN) + 0.5% sucrose + 0.05% KOH
Gluten (0.5%) + sucrose (0.5%)	0.5% Wheat gluten + 0.5% sucrose + 0.05% KOH
Soluble starch (0.5%) + sucrose (0.5%)	0.5% Soluble starch (Fisher)+ 0.5% sucrose
Sucrose (1%)	1% Sucrose
DM3	Phage applied in DI water, plants were inoculated with 10^6 CFU/ml DM3 bacteria 1 h after phage application

Table 3-2-continued

Name	Ingredients
TL 9	phage applied in DI water, plants were inoculated with 10^6 CFU/ml TL9 bacteria 1 h after phage application
Non-formulated	Deionized water only
	July 28, 2001 experiment
Casecrete (0.5%)+Sucrose (0.5%) + PCF (0.25%)	0.5% Casecrete + 0.5% sucrose+ 0.25% PCF
Skim milk (0.5%) + sucrose (0.5%)	0.5% Dehydrated skim milk + 0.5% sucrose
Skim milk (0.5%)	0.5% Dehydrated skim milk

Field experiments. The field experiments were conducted at the Horticultural Research Unit of the University of Florida in Gainesville, Florida, in 2000. Seedlings of VF 36 tomato variety were planted in a randomized complete block experimental design with six treatments and three replications per treatment. There were ten plants in a plot. The field consisted of six rows and there were three plots in every row. There was 1.5 m between rows, 2 m between plots in the same row and 51 cm between plants in the same plot. The plants were cultured according to standard horticultural methods used in the area with drip irrigation and plastic mulch (Maynard et al., 2001). The overhead irrigation in the November 15, 2000 experiment was done with two sets of four sprinklers. For data assessments six leaflets were collected from each plot.

Greenhouse experiments. Experiments were conducted in a greenhouse in Gainesville, Florida. 'Bonny Best' seedlings were grown in 10-cm pots in Terra-Lite® agricultural mix (Scott Sierra Horticultural Products Co., Marysville, OH). The plants were grown to the 4-5-leaf stage in the greenhouse at 23-28 °C, fertilized and watered as needed, and then used for phage treatment. Each treatment was applied to three plants. Following the application of treatments, the plants were arranged in a completely randomized block

design. In the March 22 and 28, 2001 experiments overhead irrigation was simulated by directing a hand-held sprinkler upwards, so that the falling drops would reach the plants. For data assessments three leaflets were collected from each plant.

Data collection and processing. At designated time intervals, leaf samples were collected from the treatments. Each sample consisted of 3-6 terminal leaflets that were located on exposed positions of the plants. The leaflets were placed into zipper-seal plastic freezer bags (The Glad Products Co®, Pittsburgh, PA). The bags were sealed, placed into a portable ice cooler and transferred to the laboratory. In the laboratory each bag was weighed, and then 100 ml deionized water was poured into each bag. The bags were shaken on a Wrist Action shaker (Burrel Co., Oakland, CA) for 15 min and then 1 ml of the rinsate was transferred to 1.5 ml microcentrifuge tubes. Then 100 µl chloroform was added to each microcentrifuge tube and the tubes were incubated on a rotary shaker for 30 min. Following incubation the chloroform was pelleted by a pulse-spin in a microcentrifuge and 700 µl of the supernatant was transferred into a sterile microcentrifuge tube. The tube was centrifuged for 15 minutes at 14000 rpm in order to remove cellular debris. The supernatant was used for phage recovery and enumeration of the phage titer.

Phage recovery. The 24-h-old bacterial culture was removed from the agar plates and suspended in sterile tap water. A 100-µl aliquot of the phage suspension was placed in a sterile 90-mm diameter Petri dish (Fisher Scientific Co., Fair Lawn, NJ) followed by the addition of 100 µl of the suspension of the host bacterium (strain BD2). Finally, 16 ml of NYA medium (0.8% (wt/V) nutrient broth; 0.6% (w/V) Bacto Agar and 0.2 % (wt/ V) yeast extract (Difco Becton Dickinson and Co., Sparks, MD)), heated to 48-50°C, was

added. The medium was swirled in the Petri dish to facilitate thorough mixing of bacterial cells and the phage virions. The plate was incubated at 28 °C for 2-3 days until the bacterial lawn appeared and the plaques were observed.

Enumeration of phage titer and data analysis. Following phage recovery, the plaques were counted at the suitable dilutions. The phage titer was expressed as number of plaque forming units (PFU) per gram leaf tissue by the following equation: y = plaque number x 1000 (since 100 µl of the original 100-ml volume was plated) / dilution ratio/ (sample bag weight - empty bag weight [g]). For statistical analysis the data acquired from each sample were transformed using log transformation ($z=\log_{10}(y+1)$), and then the data were statistically analyzed using Duncan's multiple range test by SAS System for Windows program release 8.02 (Cary, NC).

Preliminary screening. Potential protective materials (Table 3-1) which were most likely to be suitable for agricultural use were screened in an effort to reduce the number of treatments for later field and greenhouse trials. Initially, the approximate cost of the application of these materials was assessed. In a subsequent investigation we tested a casein-based (0.5%(w/v) Casecrete+ 0.25%(v/v) ammonium-zirconium-carbonate (AZCote5800M 30%, Hopton Technologies Inc., GA), tryptophan (1 PPM tryptophan (Sigma)), n-propyl-gallate (1 PPM n-propyl-gallate (Sigma)), activated carbon (2.5% Carbon (Charcoal, Mallinckrodt Inc., KY) + 0.01%(v/v) Agrimax 3 (ISP Technologies Inc., NJ)), and an alkaline gluten (1%(w/v) wheat gluten (Sigma) + 0.05%(w/v) KOH (pH 11)) formulation in two field experiments.

Results

Preliminary screening. Preliminary investigation uncovered that catalase and Tinopal LPW were prohibitively expensive at the reported effective concentration for agricultural purposes. In preliminary field tests the activated carbon significantly reduced the phage residual activity; the carbon formulated phage populations suffered 75% more reduction than the non-formulated ones in a 6 h period. The alkaline gluten formulation performed the best, while casein, tryptophan and n-propyl-gallate also enhanced phage longevity to some extent; the use of these formulations increased the longevity of phage populations in a 6 h period by 143, 44, 73 and 21%, respectively.

Comparison of alkaline gluten formulations. In preliminary field screening, the alkaline gluten formulation emerged as a good candidate for investigation. Therefore in the first experiment (July 2000) we tested several putative protective agents (Congo Red, tryptophan, n-propyl-gallate and all three together) in addition to alkaline gluten formulation (Table 3-2).

The non-formulated phage population decreased less than 10-fold overnight, but it suffered a 50-fold drop the next morning (Figure 3-1). The population did not decline further in the afternoon hours suggesting that surviving virions were located in protected pockets. The alkaline gluten formulation did not influence the nighttime reduction; however, during the day it significantly slowed down the degradation in population. Thus, by noon the gluten-formulated phage population was 20 times higher than the non-formulated population.

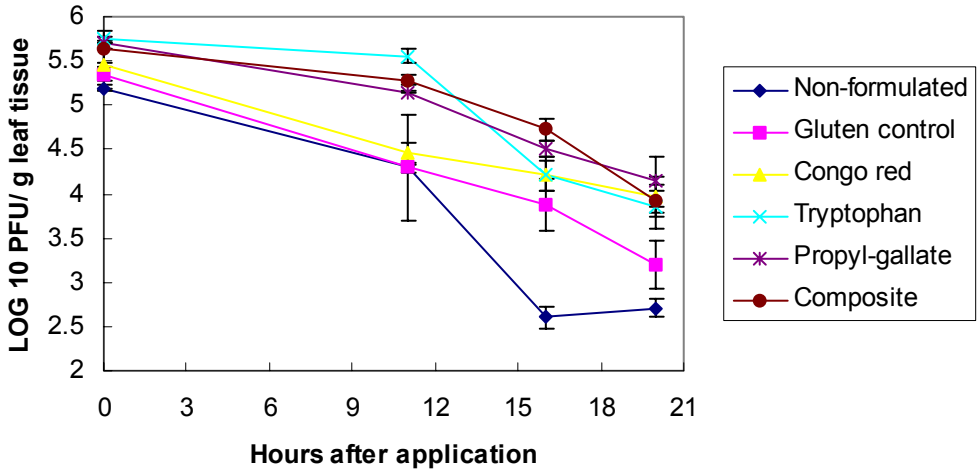

Figure 3-1. Recovery of bacteriophages from tomato leaf surface. The different formulations were sprayed on tomato plants on July 6, 2000 at 8 pm. The error bars indicate the standard error. The constitution of treatments was: gluten control – alkaline gluten (1%); congo red – gluten (1 %) + Congo Red dye (0.1%); tryptophan – gluten (1 %) + tryptophan (0.1%); propyl-gallate – gluten (1 %) + n-propyl-gallate (0.1%); composite – gluten (1 %)+ Congo Red dye (0.1%) + tryptophan (0.1%); n-propyl-gallate (0.1%); non-formulated – deionized water only.

The addition of Congo Red to alkaline gluten further reduced the decline in population during daytime, and similarly the n-propyl-gallate and tryptophan additives made a positive difference on phage longevity. The addition of Congo Red, n-propyl-gallate or tryptophan to the alkaline gluten formulation all resulted in significantly higher phage populations than the gluten formulation only 20 h after application. These three treatments were not significantly different from each other or from the composite treatment, which included all three additives, indicating that the protective effect of these substances is not additive.

Comparison of gluten, casein and lignite formulations. In the next set of field experiments (October, 2000) we tested a Na-lignate formulation, two variations of

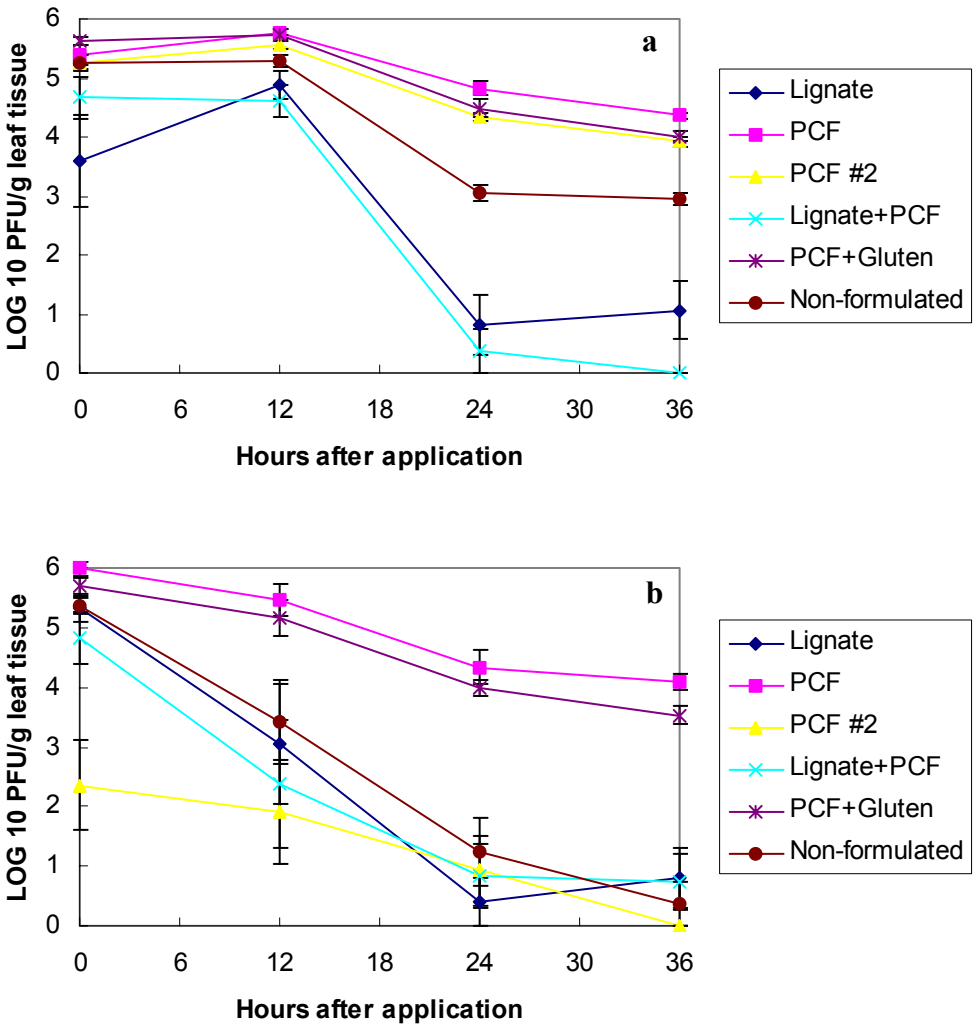

Figure 3-2. Recovery of bacteriophages from tomato leaf surface. The formulations were applied on tomato plants on **a.)** October 15, 2000 at 7 pm and **b.)** October 25, 2000 at 7 pm. The error bars indicate the standard error. The constitution of treatments was: lignate – Na-lignate (0.5%) + CaCl2 (0.05%); PCF – pregelatinized corn flour (0.5%) + sucrose (0.5%); PCF #2 – pregelatinized corn flour (0.5%) + sucrose (0.5%) + lactic acid (0.005%) + corn oil (0.15%) + isopropanol (10%); lignate + PCF – Na-lignate (0.3%) + CaCl2 (0.03%) + pregelatinized corn flour (0.2%) + isopropanol (10%); PCF + gluten – gluten (0.25%) + PCF (0.25%); non-formulated – deionized water only.

Pregelatinized Corn Flour (PCF) formulation, the combination of Na-lignate and PCF and the combination of gluten and PCF (Table 3-2). Two experiments were conducted and

results are shown in Figure 3-2. The Na-lignate formulation surprisingly decreased the phage residual activity. The PCF and PCF+gluten formulations both significantly enhanced the phage longevity, compared to the non-formulated phage, and by the end of 36 h PCF alone was more effective than PCF+gluten. The PCF #2 formulation was significantly better than the control in the first experiment (Figure 3-2a), but in the second one the phage population was greatly reduced (Figure 3-2b).

Comparison of PCF, Casein and Silwet formulations. In the next field experiment, we tested casein and Silwet, a commercially used surfactant, alone and in combination with PCF (Table 3-2). The treatments were applied in the evening just before sunset. During the first night the plots were subjected to overhead irrigation for 4 h. Phage populations in the control plots were reduced 1000-fold during the first 12-h period, primarily due to irrigation wash-off (Figure 3-3). Phage populations treated with casein, PCF and PCF+casein formulations suffered 100-fold reductions during the same time period, while the two formulations containing Silwet L-77® (Loveland Industries Inc.), had a 10-fold reduction in phage populations. No significant reduction occurred in the next 24 h for any of the formulated treatments.

Comparison of PCF, Casecrete, BSA and gelatin formulations. During spring 2001 several additional compounds were tested for their ability to enhance phage residual activity and reduce leaching. These experiments were performed in the greenhouse. Since casein increased the phage residual activity at least as much as the PCF formulation in the previous field experiment, other casein products were selected for testing. Casecrete ™ NH-400 is a commercially available casein polymer that is water soluble at neutral pH, in contrast with casein that becomes water soluble only under alkaline conditions.

Therefore, it is a better candidate for agricultural applications than casein itself. Casecrete was tested at 0.5% (w/V) alone, and in combination with PCF (0.25% Casecrete + 0.25% PCF). Gelatin and bovine serum albumin (BSA) were also tested (Table 3-2). Gelatin is used for formulation of biological control agents and behaves as a sticker, whereas BSA is used in laboratories for protecting proteins. Thus it was speculated that the buffer compound could also reduce phage degradation on the plant surface.

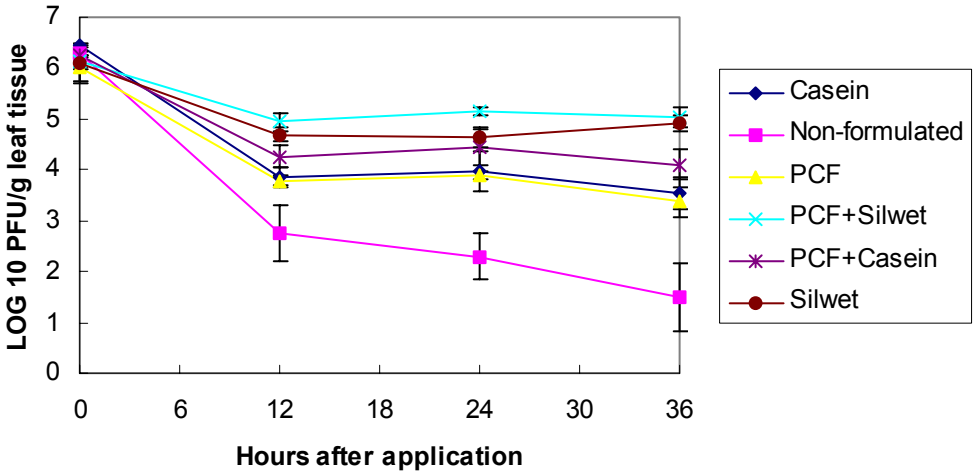

Figure 3-3. Recovery of bacteriophages from tomato leaf surface. The formulations were sprayed on tomato plants on November 15, 2000 at 7 pm. The error bars indicate the standard error. The constitution of treatments was: casein – casein (0.5%); non-formulated – deionized water only; PCF – pregelatinized corn flour (0.5%) + sucrose (0.5%); PCF + Silwet – pregelatinized corn flour (0.5%) + sucrose (0.5%) + Silwet L-77 (0.1%); PCF + casein –pregelatinized corn flour (0.25%) + sucrose (0.5%) + casein (0.25%); Silwet – Silwet L-77 (0.1%).

In these experiments the tomato plants were separated into two groups following phage application; one group was subjected to overhead irrigation and the other to drip irrigation. The surface phage populations were enumerated 4 days after application. The overhead irrigation caused a 1000-fold reduction in plots treated with non-formulated

phage compared to the drip irrigated plants (Table 3-3). The addition of gelatin provided complete wash-off protection; however, alone it did not seem to protect the phages, since

Table 3-3. Recovery of bacteriophages from tomato leaf surface 4 days after application in greenhouse

	Recovered phage population[w]	
Treatment[x]	Drip[y]	Overhead
Casecrete (0.5%)	4.1 a	2.8 a
Casecrete (0.25%) + PCF (0.25%)	3.5 ab	2.8 a
BSA[z] (0.1%)	4.1 a	2.8 a
Gelatin (0.5%)	2.5 c	2.4 a
Non-formulated	3.2 bc	0.6 b

[w] $\log_{10}$ PFU/ g leaf tissue. The means followed by different letters within a column are significantly different according to Duncan's multiple range test, P=0.05 level. The formulations were sprayed on tomato plants on March 22, 2001 at 7 pm and the plants were kept in greenhouse.
[x] All treatments contained ~10^8PFU/ml bacteriophage mixture.
[y] Drip= drip irrigation; overhead=overhead irrigation.
[z] Bovine serum albumin.

Table 3-4. Recovery of bacteriophages from tomato leaf surface 4 days after application in greenhouse

	Recovered phage population[w]	
Treatment[x]	Drip[y]	Overhead
Casecrete (0.5%) + Sucrose (0.5%) + PCF[z] (0.25%)	5.1 a	4.3 a
Casecrete (0.5%) + Sucrose (0.5%)	5.1 a	4.1 a
Casecrete (0.5%)+ PCF (0.25%)	4.3 b	3.9 a
Casecrete (0.5%)	3.4 c	3.3 b
Non-formulated	3.4 c	2.3 c

[w] $\log_{10}$ PFU/ g leaf tissue. The means followed by different letters within a column are significantly different according to Duncan's multiple range test, P=0.05 level. The formulations were sprayed on tomato plants on March 28, 2001 at 7 pm and the plants were kept in greenhouse.
[x] All treatments contained ~10^8PFU/ml bacteriophage mixture.
[y] Drip= drip irrigation; overhead=overhead irrigation.
[z] Pregelatinized corn flour.

on the drip-irrigated plants the gelatin-formulated phage population was numerically lower than the non-formulated population. In overhead-irrigated plots, where BSA, Casecrete, Casecrete+PCF and gelatin formulations were used, populations were significantly higher than the control population, while in the case of drip irrigation only the BSA and Casecrete were significantly better than the non-formulated phage.

Comparison of Casecrete formulations. In this experiment 0.5% Casecrete was tested in all combinations with PCF and sucrose in the greenhouse (Table 3-2). The addition of both materials significantly increased the phage longevity regardless of irrigation type, and the treatments containing sucrose had significantly higher phage populations where drip irrigation was used (Table 3-4). The addition of both the sucrose and the PCF did not produce significantly better results than the addition of sucrose alone.

Comparison of casein formulations. In the June 15, 2001 greenhouse experiment Casecrete formulation was compared with two other similar materials, skim milk powder and casein extract, by applying at the same concentration (Table 3-2). Gelatin was also included in the test in combination with skim milk.

As the results indicate (Table 3-5), the skim milk formulations performed significantly better than the Casecrete. The casein performed similarly to the Casecrete; however, the plants that were treated with casein developed necrotic lesions 3 days after application. The addition of gelatin to the skim milk formulation significantly reduced the phage residual activity suggesting, in accordance with the March 22, 2001 results (Figure 3-4), that gelatin had a negative effect on phage longevity.

Comparison of casein-based and flour-based formulations. In the next greenhouse experiment on June 28, 2001 several flour-based formulations and casein-based

Table 3-5. Recovery of bacteriophages from tomato leaf surface 3 days after application in greenhouse

Treatment[x]	Recovered phage population[y]
Skim milk (0.5%) + gelatin (0.5%)	3.6 c
Skim milk (0.5%)	5.4 a
Casein[z] (0.5%)	4.4 b
Casecrete (0.5%)	3.9 bc
Non-formulated	0.0 d

[x] All treatments contained ~10^8PFU/ml bacteriophage mixture.
[y] $\log_{10}$ PFU/ g leaf tissue. The means followed by different letters within a column are significantly different according to Duncan's multiple range test, P=0.05 level. The formulations were applied on tomato plants on June 15 2001 and the plants were kept in greenhouse.
[z] The casein formulation was prepared in basic solution (pH11).

formulations were tested (Table 3-2). The flour-based materials (soluble starch, gluten, all purpose flour, PCF, finomliszt and Miragel) were applied in combination with sucrose, while the Casecrete, casein and skim milk were applied alone and in combination with sucrose. Casecrete was also tested in combination with sucrose and PCF and also at a higher concentration (0.75 % vs. 0.5%). Additionally, two avirulent T3 strains (TL 9 and DM3) were tested as well as sucrose alone. The sucrose formulation (1%) had a significant effect on phage survival and was better than several flour-sucrose formulations (Table 3-6). All flour-based formulations performed significantly worse than the casein-based ones. Among them the gluten and PCF formulations were the best. Casein and skim milk were significantly better than Casecrete, when applied alone, but the addition of sucrose reduced the differences between them. Repeated applications of casein resulted in necrotic lesions on the tomato plants; thus, it appears that casein is harmful to the plants. Neither the addition of 0.25% PCF nor the 0.25% increase in

Casecrete concentration improved the effectiveness of Casecrete + sucrose. Both bacteria, TL 9 and DM 3, significantly increased the residual activity compared to the control.

Table 3-6. Recovery of bacteriophages from tomato leaf surface 2 days after application in greenhouse

Treatment[x]	Recovered phage population[y]
Casecrete (0.5%)+sucrose (0.5%) +PCF (0.25%)	5.6 ab
Casecrete (0.75%) + sucrose (0.5%)	5.7 ab
Casecrete (0.5%) + sucrose (0.5%)	6.0 a
Skim milk (0.5%) + sucrose (0.5%)	6.0 a
Casein (0.5%) + sucrose (0.5%)	5.9 a
Casecrete (0.5%)	5.2 abc
Skim milk (0.5%)	5.6 ab
Casein (0.5%)	5.7 ab
PCF (0.5%) + sucrose (0.5%)	4.6 cd
Miragel (0.5%) +sucrose (0.5%)	3.4 e
Finomliszt (0.5%) + sucrose (0.5%)	4.3 cde
All purpose flour (0.5%) + sucrose (0.5%)	3.8 de
Gluten (0.5%) + sucrose (0.5%)	5.0 bc
Soluble starch (0.5%) + sucrose (0.5%)	4.3 cde
Sucrose (1%)	3.9 de
DM3	5.7 ab
TL 9	3.7 e
Non-formulated	1.0 f

[x] All treatments contained ~10^8PFU/ml bacteriophage mixture.

[y] $\log_{10}$ PFU/ g leaf tissue. The means followed by different letters within a column are significantly different according to Duncan's multiple range test, P=0.05 level. The formulations were applied on tomato plants on June 28 2001 and the plants were kept in greenhouse.

Comparison of Casecrete and skim milk in the field. We decided to compare the skim milk formulation with the Casecrete under field conditions, because it emerged as a consistently good candidate in greenhouse tests. The 0.5% Casecrete + 0.5% sucrose + 0.25% PCF formulation, which was used in the 2001 spring field disease control trial (Chapter 5), was compared with 0.75% skim milk and 0.75% skim milk + 0.5% sucrose

formulations (Table 3-2). Skim milk applied alone was significantly more effective than the Casecrete formulation, and the addition of sucrose significantly increased the phage residual activity (Table 3-7).

Table 3-7. Recovery of bacteriophages from tomato leaf surface 24 h after application in field

Treatment[x]	Recovered phage population[y]
Casecrete (0.5%)+sucrose (0.5%) + PCF (0.25%)	4.8 c
Skim milk (0.75%) + sucrose (0.5%)	5.5 a
Skim milk (0.75%)	5.2 b

[x] All treatments contained ~10^8PFU/ml bacteriophage mixture.
[y] $\log_{10}$ PFU/ g leaf tissue. The means followed by different letters within a column are significantly different according to Duncan's multiple range test, P=0.05 level. The formulations were applied on tomato plants on July 28, 2001 on the field.

Discussion

The spray application of bacteriophages for disease control has been shown to be successful, or at least promising, in several disease management systems (Saccardi, 1993; Flaherty, 2000); however, it became clear that the degradation of phage virions was rapid during the day. Therefore, the phages have to be applied in the evening in order to achieve good disease control. The use of formulations that reduce the pace of phage degradation offers a solution for achieving effective disease control without the inconvenient nighttime application. The objective of this research was to develop such protective formulations.

Based on results of the experiments, phages disappear from the foliage within two days under field condition if applied without protective agents. The use of such agents slowed down the reduction rate up to 1000-fold in a 24-h period and increased phage

residual activity for several days. The addition of the surfactant Silwet L-77 enhanced the rain fastness of phages without interfering with their activity.

After a series of tests two groups of materials emerged that effectively increased phage residual activity after spray application on tomato foliage. These were flour- and casein-based formulations. Both types of formulations increased in efficacy when applied in combination with sucrose. Three formulations identified in this research were recommended for disease control trials:(i) PCF (0.5% PCF + 0.5% sucrose); (ii) Casecrete (0.5% Casecrete + 0.5% sucrose + 0.25% PCF) and (iii) skim milk (0.75% powdered skim milk + 0.5% sucrose). These formulations increased the concentration of phage populations 4,700-, 38,500- and 100,000-fold, respectively, 2 days after application (Table 3-6). Additionally, these formulations contain cheap, readily available, non-toxic ingredients. However, to evaluate the cost effectiveness of the use of such protective formulations for large-scale phage applications it is important to determine the ratio between their potential in increasing the efficacy of phage treatment for disease control and the additional expense of their incorporation into the formulation.

CHAPTER 4
GREENHOUSE TRIALS

Bacteriophages have been shown to control bacterial spot on tomato; however, the short residual activity of the viral control agents hindered the efficacy of phage treatment (Flaherty et al., 2000). Therefore, increasing phage longevity in the field could result in more effective disease control. Three formulations were identified (Chapter 3) that significantly increased phage residual activity in the greenhouse and the field: (i) PCF (0.5% PCF + 0.5% sucrose); (ii) Casecrete (0.5% Casecrete + 0.5% sucrose + 0.25% PCF) and (iii) skim milk (0.75% powdered skim milk + 0.5% sucrose). Several experiments were conducted to test this hypothesis. The objectives were: (i) to determine if any of the three formulations would improve the disease control efficacy compared to the non-formulated phage application; (ii) to evaluate which of the formulations is most suitable for greenhouse application; (iii) to test if lower phage concentration results in significant levels of disease control; (iv) to examine the effect of copper-mancozeb, the chemical bactericide that is widely used for controlling bacterial spot on tomato, on the efficacy of phage application; and (v) to test if different phage mixtures have different control efficacy.

Materials and Methods

Bacterial culture. *Xanthomonas campestris* pv. *vesicatoria* strain BD2 (tomato race 3) was used for inoculation in all experiments except in the June 12, 2001 experiment, where 91-118 (race T3) was used. The bacteria were grown at 28°C on nutrient agar

(0.8% (wt/V) nutrient broth (BBL, Becton Dickinson and Co., Cockeysville, MD) and 1.5% (wt/V) Bacto Agar (Difco, Becton Dickinson and Co., Sparks, MD)).

Storage and preparation of bacteriophage treatments. The phage mixtures were provided by Dr. Lee Jackson (AgriPhi Inc., Logan, UT). Mixtures A and C contained six phages active against *Xanthomonas campestris* pv. v*esicatoria* T3 strains, whereas mixture B contained five phages active against the same race. Mixtures A and B were used in the June 12, 2001 experiment, while mixture C was used in all other experiments. The phage mixtures were stored at 4°C until use. During preparation of the treatments the phage mixtures were diluted with deionized water to achieve the desired concentration. In preparation of the skim milk treatment 0.75% (w/V) dehydrated skim milk (Difco Laboratories, Detroit, MI) and 0.5% (w/V) sucrose (Mallinckrodt Inc. Paris, KY) were added to the phage suspension; in preparation of the PCF treatment 0.5% (w/V) pregelatinized corn flour PCPF 400 (Lauhoff Grain Co., Danville, IL) and 0.5% (w/V) sucrose were added, while in preparation of Casecrete treatment 0.5% (w/V) Casecrete ™ NH-400, (American Casein Company, Burlington, NJ), 0.5% (w/V) sucrose and 0.25% (w/V) PCF were added. The copper-mancozeb treatment consisted of 3.2 g/l Kocide 2000 (Griffin Corporation, Valdosta, GA) + 2.5 g/l Manzate 75DF (Griffin). The copper-mancozeb + skim milk treatment was made using the above-mentioned copper-mancozeb solution in the preparation of the skim milk formulation.

Plant material and cultivation in the greenhouse. 'Bonny Best' seedlings were grown in 10-cm pots in Terra-Lite® agricultural mix (Scott Sierra Horticultural Products Co., Marysville, OH). The plants were grown to the 4-5-leaf stage in the greenhouse at 23-28 °C, fertilized and watered as needed, and used for inoculation.

Bacterial inoculation and application of treatments. The treatments were applied using hand-held sprayers. Five plants were used for each treatment, except in the June 12, 2001 experiment, where only three plants were used. Two h after the application of treatments the plants were inoculated with the pathogen. The bacterium was grown 24 h on agar plates. The bacterial cells were suspended in sterile tap water and adjusted to A_{600}=0.3, which is approximately 10^8 CFU/ml. The bacterial suspension was misted on tomato plants using a hand-held sprayer. Following inoculation, each plant was sealed with a plastic bag in order to maintain high relative humidity that is necessary for successful bacterial colonization, and was placed in a growth chamber at 28°C and 16 h photoperiod for 48 h. After the incubation period the plastic bags were removed and the plants were transferred to the greenhouse where they were arranged in a completely randomized block design.

Data collection and analysis. The data were collected 2 weeks after inoculation. The number of lesions was assessed on five leaflets on three leaves on each plant. For statistical analysis the data acquired from each leaflet were transformed using log transformation (y=log10 (x+1)), and then the log values were averaged for each plant. The data were then subjected to an analysis of variance (ANOVA) followed by determining differences between treatment means by Duncan's multiple range test using SAS System for Windows program release 8.02 (Cary, NC).

Results

The effect of phage formulations on control of bacterial spot development. The PCF, Casecrete, skim milk-formulated and the non-formulated phage treatments were compared in two experiments (November 5 and December 3, 2001). Additionally, in the

second experiment the skim milk and the Casecrete formulations were applied without phage mixture in order to demonstrate that the formulations had no bactericidal effect. Only the skim milk + phage treatment reduced the disease development significantly in both experiments, whereas the Casecrete + phage and the PCF + phage treatments contributed to significant reduction only in one of the two experiments (Table 4-1). On plants that were treated with the skim milk and Casecrete formulations without phage and with the non-formulated phage the lesion number was not reduced.

The effect of bacteriophage concentration and copper-mancozeb co-application on control of bacterial spot development. As a result of skim milk performing very well in the previous experiments this formulation was used to examine the effect of the phage concentration on disease control efficacy. In this test three concentrations (10^8, 10^6 and 10^4 PFU/ml) of phage were applied in skim milk formulation, along with non-formulated phage containing 10^8 PFU/ml viral particles. Two other treatments were included in the test. These were copper-mancozeb and a mixture of copper-mancozeb and skim milk. For the latter treatment the copper-mancozeb solution was used in place of water in the preparation of the skim milk formulation that contained 10^8 PFU/ml phage.

The untreated plants had the highest number of lesions, but the use of non-formulated phage and the skim milk formulation containing 10^4 PFU/ml phage did not reduce the lesion number significantly (Table 4-2). The use of both the 10^6 and 10^8 PFU/ml phage resulted in a similar level of reduction, but was a significantly lower reduction than the copper-mancozeb treatment. The co-application of skim milk-formulated phage and copper-mancozeb further reduced the lesion number and was significantly more effective than all other treatments.

Table 4-1. Effect of phage formulations on bacterial spot disease development on tomato

Treatment	Average lesion number[x] Experiment 1	Experiment 2
Skim milk[y] (10^8 PFU/ml phage)	4.2 b	10.1 b
Casecrete (10^8 PFU/ml phage)	4.7 b	13.7 ab
PCF (10^8 PFU/ml phage)	16.2 a	10.8 b
Non-formulated (10^8 PFU/ml phage)	17.3 a	20.4 a
Skim milk (no phage)	NT	20.7 a
Casecrete (no phage)	NT	20.4 a
Untreated control	19.9 a	18.5 a

[x] Average lesion number per leaflet 14 days after inoculation. The means followed by different letters within a column are significantly different according to Duncan's multiple range test, P=0.05 level.

[y] The constitution of treatments were: skim milk – 0.75% powdered skim milk + 0.5% sucrose; PCF – 0.5% PCF + 0.5% sucrose; Casecrete – 0.5% Casecrete + 0.5% sucrose + 0.25% PCF; non-formulated – deionized water only.

Table 4-2. Effect of phage concentration and copper-mancozeb treatment on bacterial spot disease development on tomato

Treatment	Average lesion number[x]
Skim milk[y] (10^4 PFU/ml phage)	22.1 ab
Skim milk (10^6 PFU/ml phage)	13.8 c
Skim milk (10^8 PFU/ml phage)	16.2 bc
Non-formulated (10^8 PFU/ml phage)	17.1 abc
Copper-mancozeb	5.1 d
Copper-mancozeb + Skim milk (10^8 PFU/ml phage)	2.6 e
Untreated control	23.8 a

[x] Average lesion number per leaflet 16 days after inoculation. The means followed by different letters within a column are significantly different according to Duncan's multiple range test, P=0.05 level.

[y] The constitution of treatments were: skim milk – 0.75% powdered skim milk + 0.5% sucrose; non-formulated – deionized water only; copper-mancozeb – 3.2 g/l Kocide 2000 + 2.5 g/l Manzate 75DF.

The effect of different phage mixtures on the efficacy of disease control. Two different phage mixtures, provided by AgriPhi, Inc., were tested in this experiment. Both

mixtures contained phages active against *Xanthomonas campestris* pv. v*esicatoria* T3 strains and were provided by the same company. The titer of the two mixtures was approximately the same. The major known differences between the two mixtures were that mixture A contained six phages whereas mixture B only five phages, and that mixture A was produced several months later than mixture B.

Both phage mixtures significantly reduced the lesion number, when applied at 10^8 PFU/ml. However, mixture B achieved significantly better control of the disease than mixture A (Table 4-3).

Table 4-3.Effect of different phage mixtures on bacterial spot disease development on tomato

Treatment	Average lesion number[x]
Mixture A[y](10^8 PFU/ml phage)	27.4 b
Mixture B (10^8 PFU/ml phage)	16.8 c
Untreated control	58.1 a

[x] Average lesion number per leaflet 15 days after inoculation. The means followed by different letters within a column are significantly different according to Duncan's multiple range test, P=0.05 level.

[y] Mixture A contained six phages active against *Xanthomonas campestris* pv. v*esicatoria* tomato race 3 strains, while mixture B contained five phages active against the same race.

Discussion

The use of bacteriophages has been shown to significantly reduce bacterial spot disease development on tomato; however, the rapid degradation of the viral agents limited the efficacy of the phage treatment and created the need for frequent applications. Formulations that reduce the rate of phage degradation could contribute to more effective disease control. We previously identified three formulations, PCF, Casecrete and skim milk, that significantly increased phage longevity in the greenhouse and in the field

(Chapter 3). Our objective was to test these formulations for their ability to improve the efficacy of disease control, and to select the one that is more suitable for agricultural application. According to the results of two tests, all three formulations contributed to enhanced disease control, but the use of skim milk resulted in greater control than the PCF or Casecrete formulations. Additionally, the skim milk formulation was readily water soluble, while the other two formulations were not, thus causing difficulties during spray application.

In earlier experiments the phage treatment was used at 10^8 PFU/ ml (Flaherty et al., 2000); however, there is no indication that this is the least effective dose that contributes to disease control. Therefore, we examined if similar disease control efficacy could be achieved with lower concentration of bacteriophages. We tested 10^6 and 10^4 PFU/ml phage concentrations, and found that 10^6 PFU/ml in comparison to10^8 PFU/ml did not decrease the efficacy of disease control.

Bacterial spot is traditionally controlled with copper-mancozeb, a mixture of chemical bactericides. The hypothesis was accepted that copper-mancozeb could not be applied with phages, because it would diminish the activity of phage treatments. Thus, the farmers, who decided to manage the disease with bacteriophages, were not advised to simultaneously apply the chemical treatment. We decided to test this hypothesis so the correct recommendations could be given to the farmers in the future. We prepared the skim-milk-formulated phage treatment in copper-mancozeb solution instead of water and tested for disease control. It turned out that this formulation resulted in a synergistic effect instead of incompatibility, leading to significantly better disease control than the application of copper-mancozeb or skim milk formulated phage alone. Thus, it can be

concluded that the farmers may not have to choose between these two disease control strategies, but can use them together.

In several experiments, the use of the non-formulated phage mixture in the greenhouse did not achieve significant control of bacterial spot (data not shown), and contradicted earlier results obtained in the same greenhouse in which significant disease control was achieved (Obradovic et al., in press; Flaherty et al., 2000). The only difference between the two sets of tests was the bacteriophage mixture applied. Thus, we decided to investigate if there was a difference between the disease control efficacies of these two phage mixtures. The results of the test indicated that mixture B, used by Obradovic, was indeed more effective for disease control than the mixture A, used in our early trials (Table 4-3).

In conclusion, the use of protective formulations contributed to increased disease control efficacy of bacteriophage treatment, and the skim milk formulation was found to be most effective and the most suitable one for use in agriculture. However, the efficacy of phage depends not only on the use of such a formulation, but also on the phage mixture used for control. Thus, further research has to focus not only on the selection of better formulations but the selection of better phages for disease control, as well.

CHAPTER 5
FIELD TRIALS

Bacteriophages have been used for control of bacterial spot of tomato (Flaherty et al., 2000). However, as demonstrated in Chapter 3, the mixture applied to tomato foliage perishes rapidly under field conditions due to the effects of several harmful environmental factors, such as sunlight, rain wash-off, desiccation and high temperature. The short residual activity of the bactericidal agent seriously reduces the efficacy of phage treatment. Consequently, an increase in longevity of the viruses could result in improved efficacy of disease control.

In earlier experiments (Chapter 3) we developed formulations that significantly increased the phage residual activity on plant foliage. These formulations were: (i) PCF (0.5% PCF + 0.5% sucrose); (ii) Casecrete (0.5% Casecrete + 0.5% sucrose + 0.25% PCF) and (iii) skim milk (0.75% powdered skim milk + 0.5% sucrose).

We designed three field trials to evaluate if these formulations could enhance the efficacy of phage treatment compared to the standard, water-based phage application and compared to the standard copper-mancozeb treatment routinely used for control of bacterial spot. Additionally, we addressed the following questions: (i) whether the time of application influences the efficacy, (ii) whether the integration of phage treatment with the application of Actigard®, a systemic acquired resistance (SAR) inducer chemical compound, would lead to increased efficacy and (iii) if the increase in disease control effectiveness translates to increased fruit production.

Efficacy of the treatments was assessed by determining bacterial spot disease severity and fruit yield. Disease development was monitored by assessing lesion number and percent defoliation. For evaluation of fruit production, the total weight and the number of medium, large and extra-large fruits were assessed.

Materials and methods

Bacterial culture and inoculation. *Xanthomonas campestris* pv. *vesicatoria* strains BD2 and 91-118 (race T3) were used for inoculation in experiments 1 and 2, while in experiment 3 BD2 was used alone. The bacteria were grown at 28°C on nutrient agar medium (0.8% (wt/V) nutrient broth (BBL, Becton Dickinson and Co., Cockeysville, MD) and 1.5% (wt/V) Bacto Agar (Difco, Becton Dickinson and Co., Sparks, MD)). For inoculation, 24-h-old bacterial culture was removed from the agar plates, suspended in autoclaved tap water; the absorbance of the bacterial suspension was adjusted to OD_{600}=0.3, which resulted in a bacterial concentration of approximately 10^8 CFU/ml. Following the addition of 0.025% Silwet L-77 (Loveland Industries Inc., Greely, CO) the bacterial suspension was misted on the middle plant of each plot using a hand-held plastic sprayer.

Collection, processing and analysis of data. Two types of disease ratings were performed: (i) assessment of percentage of defoliation using the Horsfall-Barratt scale (Horsfall and Barratt, 1945) and then calculating the area under the disease progress curve (AUDPC), (Shaner and Finney, 1977) and, (ii) assessment of lesion numbers on ten terminal leaflets from each plot that were located 1-2 inches above ground. For statistical analysis the lesion count data from each plot were averaged following log transformation ($y=\log_{10}(x+1)$), and for HB rating data were averaged without transformation. The data

were subjected to an analysis of variance (ANOVA) followed by determining differences between treatment means by Duncan's multiple range test using SAS System for Windows program release 8.02 (Cary, NC). For yield data analysis, the fruits were classified into medium, large and extra-large groups according to current Florida standards and the number and weight for each class and the cull weight were recorded for each plot. The raw data were subjected to ANOVA analysis and Duncan's multiple range test.

Experiment 1. The experiment was conducted at the Horticultural Research Unit of the University of Florida in Gainesville, Florida in the 2001 spring growing season. The experiment consisted of five treatments (Table 5-1) with five replications per each treatment arranged in a randomized complete block (RCB) design. Each plot consisted of 11 plants. Seedlings of 'Mountain Fresh' tomato variety that were provided by Speedling Inc., Florida, were planted on March 23. There was 6.1 m between rows and between plots within the same row. Plants were spaced 51 cm apart within each plot. The plants were cultured according to standard horticultural methods with drip irrigation and plastic mulch (Maynard et al. 2001). The bacterial inoculation was done on April 9 early in the morning. The phage treatments (Table 5-1) contained ~10^8 PFU/ml anti-T3 and anti-T1 phage mixtures (AgriPhi Inc., UT). Both phage mixtures consisted of six phages active against bacteria of the targeted race. The treatments were applied twice weekly from April 5 to June 15 around 8 PM or around 8 AM. Disease ratings were carried out on May 7, May 21 and June 4.

Experiment 2. The experiment was conducted at the IFAS North Florida Research and Education Center in Quincy, Florida in the 2001 spring growing season concurrently with

experiment 1. ‘BHN-444’, a tomato spotted wilt virus resistant variety, was used. The seedlings were transplanted to the field on March 23. An RBC design was used with eight treatments (Table 5-1) and five replications per treatment. Each plot consisted of 11 plants. The field was made up of four rows; there was 6.1 m between the rows and between the plots in each row. Plants were spaced 51 cm apart in each plot. The middle plant in each plot was inoculated with the pathogen on April 4. The phage treatments (Table 5-1) contained ~$5x10^7$ PFU/ml anti-T1 and anti-T2 phage mixtures and were applied twice weekly from April 13 to June 18 in the evenings, around 7 PM. Disease ratings were carried out four times (May 2, May 24, June7 and June 21) during the growing season. Fruits were harvested on June 21 and 29.

Experiment 3. The experiment was conducted at the IFAS North Florida Research and Education Center in Quincy, Florida during the 2001 fall growing season. ‘BHN-555’, a tomato spotted wilt virus resistant variety, was used. The seedlings were transplanted to the field on August 3. An RBC design was used with six treatments (Table 5-1) and five replications for each treatment and 11 plants per plot. The field was composed of three rows; there was 6.1 m between the rows and between plots within rows. Plants were spaced 51 cm apart within each plot. Bacterial inoculation was done on August 6. The phage treatments (Table 5-1) were applied twice weekly from August 9 to October 18 in the evenings around 7 PM and contained ~$5x10^7$ PFU/ml anti-T1 and anti-T3 phage mixtures. Disease ratings were assessed on August 30, September 13, September 27 and October 3. Fruits were harvested twice, on October 24 and November 5. In this test the disease pressure was high, and in order to achieve comparable lesion number data, the leaflets that were assayed for lesion number were chosen from parts of the plants where

Table 5-1. Treatments used in the field experiments

Name	Information
	Experiment 1
PCF[x]	0.5% Pregelatinized corn flour (PCF) (PCPF 400, Lauhoff Grain Co. Danville, IL) + 0.5% Sucrose, applied Monday and Thursday evenings
PCF-M[x]	0.5% PCF+ 0.5% Sucrose, applied Tuesday and Friday mornings
Casecrete[x]	0.5% Casecrete + 0.5% Sucrose+ 0.25% PCF, applied Monday and Thursday evenings
Non-formulated[x]	Non-formulated phage. Phage applied in water, applied Monday and Thursday evenings
Untreated control	Untreated control
	Experiment 2
Copper-Mancozeb	3.37 kg Kocide 2000 (Griffin Corporation, Valdosta, GA) + 2.24 kg Penncozeb 75DF (Griffin) per hectare, applied once a week
PCF[x]	0.5% PCF+ 0.5% Sucrose, applied Monday and Thursday evenings
Casecrete[x]	0.5% Casecrete + 0.5% Sucrose+ 0.25% PCF, applied Monday and Thursday evenings
Actigard	3.74 g Actigard™ 50 WG (Syngenta Crop Protection, Inc. Greensboro, NC) per 100 L, applied every 14 days
PCF+Actigard	both PCF and Actigard treatments applied on the plot
Casecrete+Actigard	both Casecrete and Actigard treatments applied on the plot
Non-formulated[x]	Non-formulated phage. Phage applied in water, applied Monday and Thursday evenings.
Untreated control	Untreated Control
	Experiment 3
Copper-Mancozeb	3.37 kg Kocide 2000 + 2.24 kg Penncozeb 75DF per hectare, applied once a week
PCF[x]	0.5% PCF+ 0.5% Sucrose, applied Monday and Thursday evenings
Casecrete[x]	0.5% Casecrete + 0.5% Sucrose+ 0.25% PCF, applied Monday and Thursday evenings
Skim milk[x]	0.75% Instant Nonfat Dry Milk, fortified with vitamins A&D (Astor Products, Inc. Jacksonville, FL) + 0.5% Sucrose, applied Mondays and Thursdays in the evening
Non-formulated[x]	Non formulated phage, applied Mondays and Thursdays in the evening
Untreated control[x]	Untreated Control

[x] The treatment contained bacteriophage mixture.

the lesions did not coalesce, i.e. at the beginning of the season lower leaflets were examined, while at the end leaflets were examined towards the top.

Results

Experiment 1. Although the weather did not favor disease development and the disease pressure was low throughout the growing season, significant differences in disease development were observed among the different treatments (Table 5-2). The average lesion number on the control plants was significantly higher than on any other treatments at both dates. Non-formulated and PCF-M treatments clustered together contributing to significantly lower lesion number than the untreated control but significantly higher than PCF and Casecrete, the two formulated treatments that were applied in the evening. The Casecrete treatment was numerically most effective in reducing lesion number; however, it was not significantly better than PCF.

Disease severity, as measured by the AUDPC, was significantly higher in the control plots than in any of the other treatments (Table 5-2). The non-formulated and PCF-M treated plants had significantly less defoliation than the control but more than the PCF and Casecrete treatments. The Casecrete treatment had significantly lower AUDPC than all other treatments except PCF.

Both disease-rating methods were in agreement in showing that all phage treatments significantly reduced disease development. The two phage formulations that were applied in the evening (Casecrete and PCF) were most effective whereas the efficacy was reduced by morning application. Furthermore, they were more effective than phages without any protective agents.

Table 5-2. Effects of phage formulations and application timing on bacterial spot disease development on tomato in spring 2001 in Gainesville, FL

Treatment	Average lesion number per leaflet[x]		
	May 21	June 4	AUDPC[y]
PCF[z]	1.3c	6.4bc	46.7cd
PCF-M	3.8b	10.0b	55.5b
Casecrete	1.0c	3.9c	44.6d
Non-formulated	2.6bc	8.5b	54.1bc
Untreated control	11.0a	26.3a	63.9a

[x] The means followed by different letters within a column are significantly different according to Duncan's multiple range test, P=0.05 level.

[y] Area under the disease progress curve.

[z] The constitution of treatments was: PCF – 0.5% PCF + 0.5% sucrose, applied in the evening; PCF-M – 0.5% PCF + 0.5% sucrose, applied in the morning; Casecrete – 0.5% Casecrete + 0.5% sucrose + 0.25% PCF, applied in the evening; Non-formulated – deionized water only, applied in the evening. All treatments contained ~10^8 PFU/ml of two phage mixtures active against *Xanthomonas campestris* pv. *vesicatoria* tomato race 1 and race 3 strains, respectively.

Harvest data was not assessed because of the generally low physiological state of the plants at the end of the growing season. This was due to insect damage, diseases other than bacterial spot, and the large ratio of rotten fruits.

Experiment 2. The disease pressure was low throughout the season, except for the last few weeks before harvest. This did not allow for pronounced differences in plant health between the different treatments. Additionally, bacteriophage contamination was a problem: despite the 6.1-m distance between rows, anti-T3 phages were detected in plots that had not received phage sprays. Nevertheless, both disease incidence and severity data indicated significant differences between the treatments (Table 5-3). The control plants were exposed to the highest disease pressure, being significantly higher than any of the treated ones. The untreated control had the most lesions in all assessments, and the disease progress was significantly higher in the control plots than in plots of any other treatment. The application of copper-mancozeb, Actigard and non-formulated treatments

resulted in a similar level of disease control; these treatments achieved a significant reduction in lesion number and percent defoliation compared to the untreated control; however, they were less effective than the formulated phage treatments.

Table 5-3. Effect of phage, Actigard and copper-mancozeb treatments on bacterial spot disease development on tomato in spring 2001 in Quincy, FL

	Average lesion number per leaflet[x]				
Treatment	May 2	May 24	June 7	June 21	AUDPC[y]
Copper-Mancozeb[z]	5.5 ab	6.3 ab	2.8 bc	9.8 b	48.2 b
PCF	3.4 ab	6.0 b	2.4 bc	4.1 bc	40.9 c
Casecrete	3.8 ab	1.6 c	1.9 bc	1.8 cd	34.4 d
Actigard	7.4 ab	4.6 b	3.7 ab	3.9 bc	50.8 b
PCF+Actigard	2.5 b	3.4 bc	1.8 bc	2.1 cd	36.8 cd
Casecrete+Actigard	1.6 b	1.4 c	1.4 c	1.5 d	33.8 d
Non-formulated	7.2 ab	7.2 ab	3.2 b	6.0 b	48.4 b
Untreated control	9.8 a	12.8 a	7.3 a	25.7 a	60.3 a

[x] The means followed by different letters within a column are significantly different according to Duncan's multiple range test, P=0.05 level.

[y] Area under the disease progress curve.

[z] The constitution of treatments was: copper-mancozeb – 3.37 kg Kocide 2000 + 2.24 kg Penncozeb 75DF per hectare; PCF – 0.5% PCF + 0.5% sucrose, Casecrete – 0.5% Casecrete + 0.5% sucrose + 0.25% PCF; Non-formulated – deionized water only; Actigard – 3.74 g Actigard™ 50 WG/100 L,. PCF and Casecrete treatments contained ~$5x10^7$ PFU/ml of two phage mixtures active against *Xanthomonas campestris* pv. *vesicatoria* tomato race 1 and race 3 strains, respectively, and were applied in the evening, twice weekly. Copper-mancozeb was applied once weekly, Actigard was applied once in two weeks.

Casecrete, Casecrete+Actigard and PCF+Actigard treatments were the most effective treatments for reducing disease development, and resulted in significantly lower AUDPC and lesion number than all other treatments, except PCF. The Phage + Actigard combinations performed significantly better than Actigard alone, but only slightly better than their phage counterparts (PCF vs. PCF+ Actigard; Casecrete vs. Casecrete+ Actigard).

The harvest data (Table 5-4) were not very conclusive. Only the large fruit yield of the copper-mancozeb treated and the control plots differed significantly, copper-mancozeb being the lower one.

Table 5-4. The effect of phage, Actigard and copper-mancozeb treatments on tomato fruit production in spring 2001 in Quincy, FL

Treatment	Medium[y]	Large	Extra Large	Total marketable
Copper-Mancozeb[z]	2.8	12.1 b	47.9	62.9
PCF	4.4	17.0 ab	49.4	70.8
Casecrete	4.6	17.9 ab	52.6	75.1
Actigard	5.5	19.1 ab	55.9	80.6
PCF+Actigard	4.0	15.1 ab	49.9	68.9
Casecrete+Actigard	4.7	17.3 ab	43.4	65.3
Non-formulated	3.6	14.0 ab	45.8	63.5
Untreated control	4.2	19.5 a	54.3	78.0

[y] Tons per hectare. The means followed by different letters within a column are significantly different according to Duncan's multiple range test, P=0.05 level.

[z] The constitution of treatments was: copper-mancozeb – 3.37 kg Kocide 2000 + 2.24 kg Penncozeb 75DF per hectare; PCF – 0.5% PCF + 0.5% sucrose, Casecrete – 0.5% Casecrete + 0.5% sucrose + 0.25% PCF; Non-formulated – deionized water only; Actigard – 3.74 g Actigard™ 50 WG/100 L,. PCF and Casecrete treatments contained ~$5x10^7$ PFU/ml of two phage mixtures active against *Xanthomonas campestris* pv. *vesicatoria* tomato race 1 and race 3 strains, respectively, and were applied in the evening, twice weekly. Copper-mancozeb was applied once weekly, Actigard was applied once in two weeks.

The anti-T1 phage activity was monitored following two phage applications, on May 1 and May 22. In both cases the non-formulated phage populations practically disappeared by the morning of the third day after the application, but the use of protective formulations significantly prolonged the residual activity (Figure 5-1). The populations on the Casecrete treated plots were significantly higher than on PCF treated ones. The Actigard application did not decrease the phage populations.

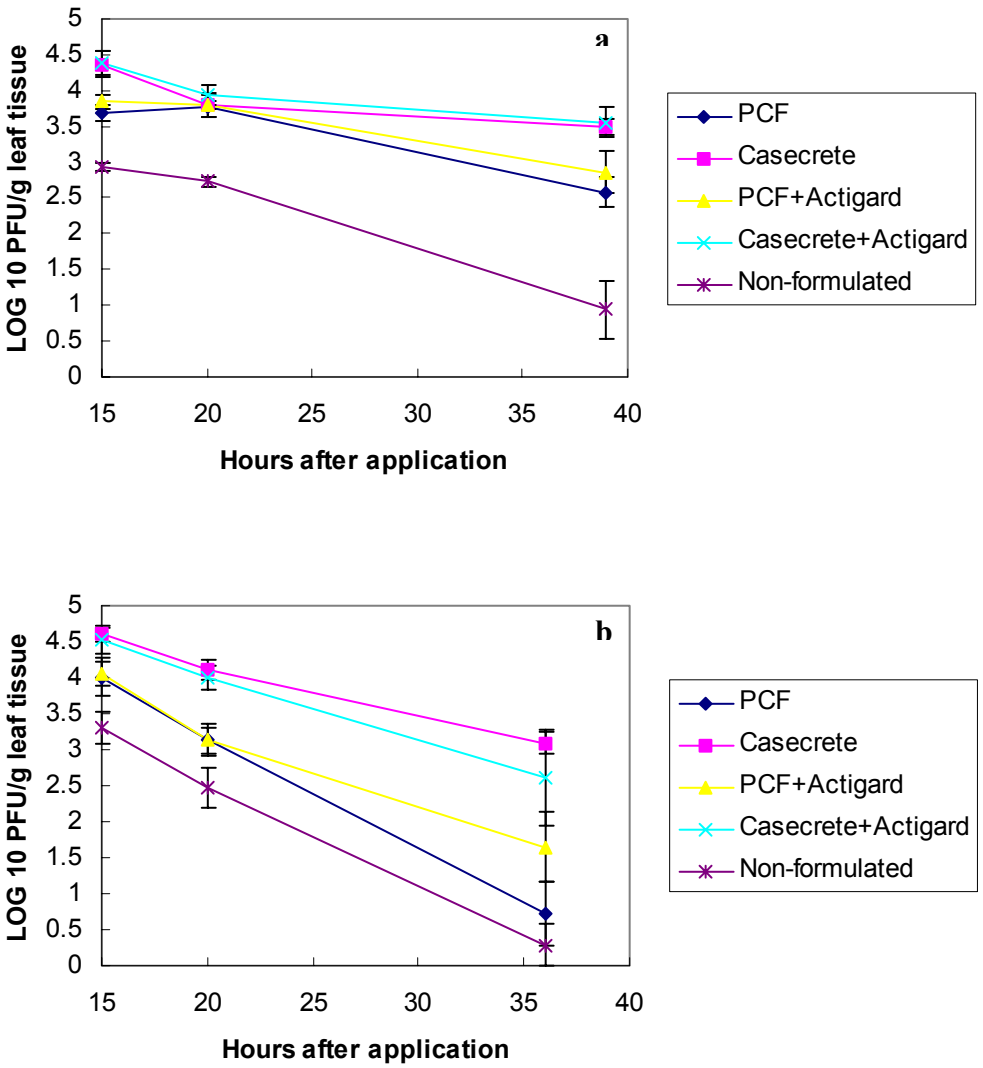

Figure 5-1. Recovery of bacteriophages from tomato leaf surface. The formulations were applied on tomato plants on a.) May 1, 2001 7:30 pm and b.) May 22, 2001 at 7 pm. The error bars indicate the standard error.

Experiment 3. This experiment was carried out in the fall growing season. The disease pressure was high, resulting in coalescing lesions and a high degree of defoliation in all treatments. The lesion count data only provided significant differences among the treatments at the beginning of the season (Table 5-5). However, general trends were noticed: untreated control, copper-mancozeb and non-formulated treatments clustered

Table 5-5. Effect of phage formulations and copper-mancozeb application on bacterial spot disease development on tomato in fall 2001 in Quincy, FL

	Average lesion number per leaflet[x]				
Treatments	Aug 30	Sept 13	Sept 27	Oct 8	AUDPC[y]
Copper-Mancozeb[z]	16.1a	18.5	1.4	9.4	158.7a
PCF	4.5bc	14.8	1.3	7.8	123.3c
Casecrete	1.9c	11.7	2.1	9.1	106.8d
Skim milk	1.5c	11.4	1.5	7.1	115.1cd
Non-formulated	4.4bc	25.6	2.2	9.3	127.9bc
Untreated control	8.8ab	21.6	2.1	11.0	140.4b

[x] Average lesion number per leaflet. The means followed by different letters within a column are significantly different according to Duncan's multiple range test, P=0.05 level.

[y] Area under the disease progress curve.

[z] The constitution of treatments was: copper-mancozeb – 3.37 kg Kocide 2000 + 2.24 kg Penncozeb 75DF per hectare; skim milk – 0.75% nonfat dry milk commercial formulation + 0.5% sucrose; PCF – 0.5% PCF + 0.5% sucrose, Casecrete – 0.5% Casecrete + 0.5% sucrose + 0.25% PCF; Non-formulated – deionized water only. All treatments, except copper-mancozeb, contained ~$5x10^7$ PFU/ml of two phage mixtures active against *Xanthomonas campestris* pv. *vesicatoria* tomato race 1 and race 3 strains, respectively, and were applied in the evening, twice weekly. Copper-mancozeb was applied once weekly.

together having higher incidence than the PCF, Casecrete and skim milk ones, which also clustered together. Results from the AUDPC distinguished between the treatments (Table 5-5). The copper-mancozeb treated plants suffered the most defoliation, and had a significantly higher AUDPC value than the control plants. The untreated control and the non-formulated phage treatment clustered together having higher incidence than the PCF, Casecrete and skim milk ones, which also clustered together. Results from the AUDPC distinguished between the treatments (Table 5-5). The copper-mancozeb treated plants suffered the most defoliation, and had significantly more AUDPC than the control plants. The non-formulated phage treatment reduced the defoliation compared to the control numerically, while all formulated phage treatments resulted in significant reduction. Casecrete and skim milk were the most effective treatments.

There were no significant differences in fruit production among the treatments (Table 5-6). The Casecrete and skim milk formulations that were the most effective for disease control did not contribute to a significant increase in fruit production. In fact both treatments slightly reduced the yield compared to the control. Interestingly, the PCF treatment, which was the least effective phage formulation for disease control, resulted in the highest yield.

In this experiment twice weekly applications of non-formulated phage did not reduce bacterial spot disease severity significantly. The use of protective formulations was necessary to achieve significant control of the disease. All phage treatments were significantly more effective in disease control than the copper-mancozeb application. Casecrete and skim milk formulations performed the best in disease control, although neither of them increased fruit production.

Discussion

Bacteriophages are currently being used for control of bacterial spot of tomato; however, the rapid degradation of viral particles on plant foliage in commercial fields limits the efficacy of phage treatment. The use of protective formulations that increase the longevity of phages could enhance the efficacy of phage treatments. Additionally, application of the viral agents in the evening could contribute to increased efficacy, since sunlight is one of the most destructive factors responsible for inactivation of phages.

Table 5-6.Effect of phage formulations and copper-mancozeb treatments on tomato fruit production in fall 2001 in Quincy, FL[x]

Treatment	Medium[y]	Large	Extra Large	Total marketable
Copper-Mancozeb[z]	11.0	21.9	25.8	58.7
PCF	12.6	24.3	28.7	65.6
Casecrete	10.6	21.5	27.5	59.6
Skim milk	9.7	22.0	25.4	57.1
Non-formulated	11.9	23.6	26.0	61.6
Untreated control	10.1	21.6	30.2	61.9

[x] Results of experiment 2.

[y] Tons per hectare. The means followed by different letters within a column are significantly different according to Duncan's multiple range test, P=0.05 level.

[z] The constitution of treatments was: copper-mancozeb – 3.37 kg Kocide 2000 + 2.24 kg Penncozeb 75DF per hectare; skim milk – 0.75% powdered skim milk + 0.5% sucrose; PCF – 0.5% PCF + 0.5% sucrose, Casecrete – 0.5% Casecrete + 0.5% sucrose + 0.25% PCF; Non-formulated – deionized water only. All treatments, except copper-mancozeb, contained ~$5x10^7$ PFU/ml of two phage mixtures active against *Xanthomonas campestris* pv. *vesicatoria* tomato race 1 and race 3 strains, respectively, and were applied in the evening, twice weekly. Copper-mancozeb was applied once weekly.

In order to test these hypotheses we implemented three field trials in which we tested three protective phage formulations: PCF, Casecrete and skim milk. These were identified for their ability to increase phage residual activity. Also, two application times were tested, evening and morning. Additionally, phage treatments were combined with Actigard, an SAR-inducing chemical agent. The results of the experiments indicated that all three protective formulations increased the efficacy of phage treatment. Casecrete and skim milk were shown to be the most effective treatments. There were problems associated with using PCF and Casecrete, which are not completely water-soluble. Application of sprays containing PCF and Casecrete were difficult to apply and may not be suitable for commercial application.

The comparison between evening and morning application showed that the evening application significantly increased the efficacy compared to morning application.

Thus the evening application time should be an emphasized part of disease management with bacteriophages.

The combined application of phage and Actigard did not increase the control efficacy compared to the phage application alone. Even though the combination of these control methods did not prove to be beneficial, the phage application could become a component of an integrated disease management strategy.

Despite the pronounced differences between the treatments for disease control, the yield data did not indicate any significant difference between treatments. This fact could be the result of widespread distribution of phages; despite the distance between rows, phages reached plots where none were applied. Furthermore, they replicated on the host. This phage contamination probably resulted in disease control even on the untreated control plots, and may have been the reason for fewer differences between treatments.

In conclusion, the findings of the field trials suggest that for best results protective formulations should be used for phage preparation before application, and they should be applied in the evening, close to sunset. For commercial application the skim milk formulation is the most promising, since skim milk powder is easily available, relatively cheap and readily becomes soluble in water; however, further testing of this formulation is necessary since it was included only in one field test.

CHAPTER 6
SUMMARY AND CONCLUSIONS

Florida provides about half of the domestic fresh-market tomato production, and the tomato is the economically most important vegetable product of the state (Florida Agricultural Statistics Service, 2001). Bacterial spot of tomato, caused by *Xanthomonas campestris* pv. *vesicatoria*, is one of the major tomato diseases in Florida and can cause up to a 50% loss in yield (Scott and Jones, 1984; Pohronezny and Volin, 1983). Management of the disease is routinely done by cultural means and by the application of copper-mancozeb, a mixture of chemical bactericides. Nevertheless, no sufficient control of the disease is possible if the environmental conditions favor the spread of the disease. (Kucharek, 1994).

Bacteriophages, viruses that infect bacteria, have been introduced for the control of bacterial spot of tomato in the 1990s. Twice weekly applications of a mixture of four host range mutant phages that were active against the causal agent of the disease resulted in significantly better disease control than the standard copper-mancozeb treatment (Flaherty et al., 2000). Additionally, the yield of extra-large fruits was significantly higher on bacteriophage-treated plants than on copper-mancozeb treated ones. However, the phage treatment was effective only when the bacteriophages were applied early in the morning, before dawn. The short residual activity of the control agents hindered the efficacy of phage treatment when applied during daytime. Bacteriophages are rapidly degraded in the field, and practically disappear from tomato foliage 2 days after application (Figure 5-1).

The use of formulations that increase the bacteriophage longevity could improve the efficacy of the phage treatment and allow more convenient application time. Additionally, it could permit reduction in phage dose and in frequency of application.

The objectives of this study were (i) to develop protective formulations, (ii) to test if the addition of these formulations to the phage mixture would improve disease control, and (iii) to investigate if the improvement manifests in increased yield.

In order to develop a protective formulation that would increase the residual activity of bacteriophages, a number of materials were identified that had been previously shown to increase the longevity of microbial biocontrol agents, such as baculoviruses and the bacterium *Bacillus thuringiensis*. These candidate materials were compared in a series of greenhouse and field tests for their ability to reduce the pace of phage degradation. Two groups of formulations turned out to be most effective, the combinations of flour and sucrose, and casein compounds. Three formulations were selected for disease control trials: (i) PCF (0.5% pregelatinized corn flour (PCF) + 0.5% sucrose), (ii) Casecrete (0.5% Casecrete NH-400 (a water-soluble casein protein polymer)+ 0.5% sucrose + 0.25% PCF), and (iii) skim milk (0.75% powdered skim milk + 0.5% sucrose). The formulations increased the concentration of phage populations 4,700-, 38,500- and 100,000-fold, respectively 2 days after the application (Table 3-7).

In greenhouse experiments all three formulations improved the disease control efficacy of bacteriophage treatment, and the skim milk was most effective (Table 4-1). In addition to its superiority in effectiveness, the skim milk formulation was easy to apply whereas the application of the PCF and Casecrete caused difficulties. The latter two formulations were not completely water-soluble, and occasionally clogged the sprayer.

The co-application of bacteriophages in skim milk formulation and copper-mancozeb resulted in significantly better disease control than with bacteriophages or with copper-mancozeb alone (Table 4-2), indicating that the copper did not inactivate the phages or that the skim milk formulation protects the phages from inactivation. Therefore, these two control methods could possibly become a part of an integrated management strategy for controlling bacterial spot in the future. However, further greenhouse and field trials are necessary before major conclusions can be drawn about co-application of these two agents.

In the three field trials all three formulations increased the efficacy of phage treatments compared to the non-formulated phage as well as compared to the copper-mancozeb application. The PCF was less effective in all three trials than Casecrete, whereas the skim milk, which was only included in one trial, performed similarly to Casecrete (Tables 5-2, 5-3 and 5-5). In these trials we also noticed the difficulty in applying PCF and Casecrete formulation. Pregelatinized corn flour, which was included in both the PCF and Casecrete formulations, is the component that is not completely water soluble and thus responsible for clogging the nozzle of the sprayer. The inclusion of pregelatinized corn flour in the Casecrete formulation did not contribute to increased phage longevity (Tables 3-5 and 3-7), so in the future it should not be included.

Our results indicated that the time of application of phage treatment could also influence the efficacy of disease control. The morning application of PCF formulation resulted in significantly reduced disease control compared to the evening application of the same formulation (Table 5-2).

The integration of Actigard, a chemical compound that induces systemic acquired resistance in plants, into the control strategy with bacteriophage treatments resulted in significantly better disease control than the Actigard application alone and was numerically although not significantly better than the phage treatments alone (Table 5-3). Thus, the integration of bacteriophages and Actigard seems promising and should be further investigated.

In contrast to the improved efficacy in disease control, none of the treatments increased the yield compared to the untreated control in those two experiments when yield was assessed (Tables 5-4 and 5-6). It is possible that the movement of the bacteriophage into the unsprayed control plots could have affected the differences. High populations of phages were detected in each plot by the end of experiment 3 indicating that the phages reached every plot where they were not applied, and replicated there on their host bacterium. Thus, even the untreated control plots were indirectly treated with phages, which could have reduced the differences between treatments.

In conclusion, our research identified several formulations that significantly increased the residual activity of bacteriophages on tomato foliage. The use of these formulations significantly increased the efficiency of bacteriophage treatment for controlling bacterial spot in greenhouse and field trials. The two most promising formulations were (i) Casecrete: 0.5% Casecrete, 0.5% sucrose and 0.25% pregelatinized corn flour, and (ii) skim milk: 0.75% powdered skim milk and 0.5% sucrose. In our trials however, the fruit yield was not increased by any of the formulations.

Additional questions remained, however. As a result of a series of trials the Casecrete and skim milk formulations were identified. Still, the optimal ratio of

ingredients within these formulations and the total ratio of the formulations compared to the water still need to be investigated. Our data indicated that the co-application of phage treatment with copper-mancozeb or with Actigard resulted in a synergistic effect. However, further examination is needed to achieve conclusive results about these phenomena. Further trials are necessary to decide between the skim milk and the Casecrete formulations, and about the possible integration of the phage treatment with chemical pesticides, such as Actigard or copper-mancozeb. The phage contamination that was detected in the field trials and possibly contributed to the inconclusive yield data by reducing the differences between treatments raises the need for an experimental design that can reduce error originating from this source.

Avirulent mutants of the bacterium were used successfully to propagate phages in a greenhouse experiment (Table 3-7). This result opens the door to a completely new strategy in which phages and bacteria are simultaneously used to establish a permanently high phage population on the field and achieve disease control. The development of such a strategy could be subject for future research.

The phage titers used in the disease control experiments in the field were 10^8 and $5x10^7$ PFU/ml. However, greenhouse results indicated that 10^6 PFU/ml achieved as good disease control as the 10^8 PFU/ml (Table 4-2). Thus, the determination of the lowest effective phage titer should be done in the future.

Our greenhouse data indicated that there was a significant difference between the efficacies of different phage mixtures. Additional research should address the improvement of the efficacy of phage mixtures themselves.

In summary, our results indicated that the efficacy of phage treatment could be increased by the use of protective formulations and by the proper timing of applications. Nevertheless, further research is needed to optimize the formulations, application frequency, phage dose and the constitution of phage mixtures so that phage treatment could stand as a valid alternative to chemical bactericides for the treatment of bacterial plant diseases in the future.

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BIOGRAPHICAL SKETCH

Botond Balogh was born to Balogh Antal and Gyuris Borbála, in Kalocsa, Hungary, on March 25, 1976. He graduated from the Ságvári Endre High School in Szeged in 1994 with a mathematics major. He attended the University of Agronomy, College of Agricultural Sciences, in Gödöllõ from 1994 to 1998, majoring in horticulture and plant breeding – genetics. He participated in a research program for pepper breeding via anther and microspore culture under the guidance of Judith Mitykó in the Agricultural Biotechnological Center, Gödöllõ, from 1996 to 1998. In 1998 he took part in a yearlong agricultural exchange program organized by the CAEP. In the summer and fall of 1999 he attended Brevard Community College in Cocoa, FL. He was admitted to the University of Florida, College of Agricultural and Life Sciences, in January 2000, and graduated with the degree of Bachelor of Science with the plant science major and plant pathology minor in August 2000. During his undergraduate studies he participated in a research project characterizing a mobile genetic element found in the genome of several strains of *Xanthomonas campestris* pv. v*esicatoria,* a plant pathogenic bacterium. He attended the graduate program of the University of Florida, College of Agricultural and Life Sciences, Department of Plant Pathology, from August 2000 to May 2002. He conducted a research project on developing strategies for improving the efficacy of bacteriophages for controlling bacterial spot of tomato under the guidance of Dr. Jeffrey B. Jones, Dr. Timur M. Momol and Dr. Stephen M. Olson.