

Journal of Biological Control, 38(4): 428-437, 2024, DOI: 10.18311/jbc/2024/43011

Research Article

Enhancing phage stability and integrated management strategies for effective biocontrol of bacterial wilt in solanaceous crops

PRADEEP MANYAM[1*] and V. B. NARGUND[2]

[1]*Deprtment of Plant Pathology, S. V. Agricultural College, Acharya N. G. Ranga Agricultural University, Tirupati – 517502, Andhra Pradesh, India*

[2]*Department of Plant Pathology, University of Agricultural Sciences, Dharwad – 580005, Karnataka, India*

**Corresponding author E-mail:* m.pradeep@angrau.ac.in

ABSTRACT: In the present study, the efficacy of three protective formulations was evaluated to enhance the stability of phages under *in vitro* conditions. A glasshouse experiment was conducted to assess their effectiveness as biocontrol agents against *Ralstonia solanacearum*, a pathogen responsible for wilt in solanaceous crops. Protective formulations for bacteriophages demonstrated significant variation in phage stability over 16 days. The highest phage population was recorded in T6 (Casecrete + Skim Milk) at 1.84 × 10^5 PFU/g on day 4, while T8 (Phage alone) had the lowest population at 0.96 × 10^5 PFU/g. Phage stability declined over time, but formulations containing Pregelatinized Corn Flour, Casecrete, and Skim Milk (T7) maintained the most consistent phage populations, reaching 1.69 × 10^4 PFU/g on day 12. Phage alone (T8) showed the lowest stability, emphasizing the need for protective agents. Phage-based treatments that were evaluated for their effectiveness in controlling bacterial wilt in tomato and brinjal exhibited that phage mix alone (T4) provided 100% disease reduction in tomato and 94.44% in brinjal. Combinations of phage mixed with other agents, such as PGPR (T5), Bleaching powder (T6), and COC and Streptocycline (T8), also showed significant disease reduction, with T8 achieving 94.11% disease reduction in tomato and 94.44% in brinjal. Chemical treatments like COC and Streptocycline were effective but performed better when combined with phage-based treatments. In the untreated control (T9), disease severity reached 85.00% in tomatoes and 90.00% in brinjal. The study conclusively demonstrates that phage formulations, particularly in combination with other biocontrol agents or chemicals, are highly effective in managing bacterial wilt in tomato and brinjal, offering a sustainable alternative to chemical control.

KEYWORDS: Bacteriophages, biocontrol, phage therapy, *Ralstonia solanacearum*, solanaceous crop

(Article chronicle: Received: 22-06-2024; Revised: 18-09-2024; Accepted: 21-09-2024)

INTRODUCTION

Ralstonia solanacearum is a soil-borne Gram-negative bacterium that is the causative agent of bacterial wilt in many important crops (Hayward, 2000; Yabuuchi *et al.,* 1995). This bacterium has an unusually wide host range of over 200 species belonging to more than 50 botanical families (Hayward, 2000). It infects roots and exhibits strong tissue-specific tropism within the host, specifically invading and extensively multiplying in the xylem vessels. With diverse virulence factors, the organism gains entry to the roots through natural or mechanically damaged openings. It then colonizes the vascular tissues, inducing both internal and external signs of distress, ultimately leading to the plant's complete wilting.

Ralstonia solanacearum, a persistent bacterium, readily travels through irrigation water in fields, establishing itself on alternative hosts for extended periods (Álvarez *et al.*, 2010; Hong *et al.*, 2008). Due to the limited efficiency of the current integrated management strategies, bacterial wilt continues to be an economically serious problem for field-grown crops in many subtropical, tropical and warm areas of the world (Hayward, 2000). The use of copper compounds and other agrochemicals, while common, carries significant environmental implications and is often associated with the development of bacterial resistance or the transition to a viable but non-culturable state (Grey & Steck, 2001; Yuliar *et al.,* 2015). Alternatively, considerable efforts have been directed towards biocontrol strategies, which offer the advantage of being environmentally sustainable. These approaches primarily include the utilization of antagonistic microorganisms, avirulent strains of pathogens, bacteriophages, *etc*. (Yuliar *et al.,* 2015; Buttimer *et al.,* 2017).

Among the advancements in biocontrol measures for bacterial wilt, there is a growing interest in bacteriophages,

the most prevalent microorganisms governing bacterial populations in the environment. Many bacteriophages exhibit a narrow host range, making them potential biocontrol agents capable of specifically targeting bacterial pathogens without posing risks to either the protected crop or the surrounding environmental microbiota. Consequently, phages offer a promising avenue for biocontrol, known as phage therapy, against bacterial wilt disease (Fujiwara *et al.*, 2011; Bae *et al.*, 2012; Bhunchoth *et al.*, 2015), with successful applications demonstrated in managing various plant diseases caused by phytopathogenic bacteria (Jones *et al.*, 2007; Balogh *et al.*, 2010; Doffkay *et al.*, 2015; Buttimer *et al.*, 2017; Carstens *et al.*, 2018, Nga *et al.*, 2021; Jain *et al.*, 2023).

In this study, therefore, we isolated two phages infecting *R. solanaceraum* isolates from environmental water and soils and demonstrated their stability through protective formulations and their effectiveness as a disease management strategy that could be used as part of integrated management options.

MATERIALS AND METHODS

The strains of *R. solanacearum* used in this study were isolated and cultured in cultured in CPG medium (Casamino acid, Peptone and Glucose) and Nutrient Broth (NB) at 28°C. Pathogenicity was proved on susceptible genotypes of tomato and brinjal.

The white pinkish irregular colonies of isolates of *R. solanacearum* grown on CPG medium for 48 h at 28°C were harvested and suspended in sterile distilled water. The OD value of the above bacterial suspension was adjusted spectrophotometrically to reach an OD of 0.3 at 600 nm (which is approximately $5x10^8$ colony-forming units (CFU)/ml). Tomato (Pusa Ruby variety) and brinjal (Manjri hybrid) bacterial wilt susceptible genotypes were grown in pots at 27°C for the pathogenicity test. The plants were inoculated at the root zone by making slight injury to the root with a disposable syringe and 5 ml of inoculum was poured into the injured portion and observed regularly for wilting symptoms.

Soil samples were collected from tomato and brinjal growing fields in Northern Karnataka. Each 10 g of soil was suspended in 50 ml of sterile distilled water and vigorously shaken for 20 min at room temperature to release bacteriophages. For pond water or municipal sewage water, an equal volume of phage broth and 1 ml of broth from a 24-h-old culture of *R. solanacearum* bacteria were added, followed by 24 h of incubation. The mixtures obtained from the various samples were then subjected to the isolation of bacteriophages using the method outlined by Smith and Huggins in 1982. Subsequently, the mixtures were centrifuged at 10,000 rpm for 15 min to separate the supernatant, which was later filtered through 0.22 µm pore size filters to remove bacteria. The Plaque Forming Units (PFU) assay was conducted using the double-layer agar diffusion method. Phages were further purified through three subsequent single plaque isolations using phage lysate buffer (SM buffer).

Protective formulations on bacteriophage population density

To assess the abundance and availability of bacteriophages in soil within the rhizosphere of only tomato plants affected by bacterial wilt, a combination of the above two bacteriophages was introduced to the soil. The application of this phage mixture was conducted both with and without protective formulations (list of formulations used is given in Table 1). Tomato seedlings of the susceptible Pusa Ruby variety were raised in a nursery and transplanted after three weeks for the experiment. The bacteriophages, treated with chloroform (helps to kill host bacteria in phage suspensions leaving isolated phages because chloroform acts on the lipid layer of bacteria which is absent in phages) supplemented with protective formulations, were administered at a concentration of approximately 10^9 PFU per ml (the phage title was calculated by double agar plaque assay method by serial dilution). Each small plastic pot containing sterilized soil, previously confirmed to exhibit no phage activity against *R. solanacearum,* was applied with 20 ml of the phage mixture formulations as drenching near the root zone. The bacterial suspension was adjusted to achieve an Optical Density (OD) of 0.26 at 600 nm, which was determined to contain roughly 10^8 Colony-Forming Units (CFU) per ml. Seedlings were inoculated at the root zone by creating minor injuries, which were then immersed in the bacterial suspension for one minute before being promptly transplanted into the phage-treated pots. Additionally, 5 ml of the bacterial inoculum was poured onto the injured portion to increase the disease pressure. Each treatment was replicated five times. Phage population density was evaluated using a double agar layer assay from soil samples, without enrichment with the host bacterial strain. Enrichment procedure is generally followed for initial isolation of phages as their concentration is less and the current study focused on assessing active phage concentration in inoculated soils. The study monitored phage population densities at intervals of 4, 8, 12, and 16 days after the treatment of the phage formulations.

To study the effect of bacteriophage formulations in reducing bacterial wilt disease development, the Per cent Disease Index (PDI) was calculated in the above-mentioned treatments after 35 days of post-inoculation of protective formulations. The PDI was calculated as methodology described in the next experimental section.

Table 1. Evaluation of bacteriophage formulations on phage population density in tomato wilt

S. No.	Treatment details	4 DAS	8 DAS	12 DAS	16 DAS
		Phage population (x 10^5 PFU/g)	Phage population (x 10^5 PFU/g)	Phage population(x 10^4 PFU/g)	Phage population (x 10^4 PFU/g)
T_1	Pregelatinized corn flour	1.65 (5.22)*	0.72 (4.86)	1.33 (4.12)	0.38 (3.58)
T_2	Casecrete	1.25 (5.10)	0.46 (4.66)	1.54 (3.85)	0.63 (3.80)
T_3	Skim milk	1.14 (5.06)	0.53 (4.73)	1.04 (4.01)	0.46 (3.66)
T_4	Pregelatinized corn flour + Casecrete	1.31 (5.12)	0.80 (4.90)	1.27 (4.10)	0.56 (3.75)
T_5	Pregelatinized corn flour+ Skim milk	1.42 (5.15)	0.63 (4.80)	1.00 (4.00)	0.29 (3.46)
T_6	Casecrete + Skim milk	1.84 (5.27)	0.62 (4.79)	1.26 (4.10)	0.60 (3.77)
T_7	Pregelatinized corn flour + Casecrete + Skim milk	1.67 (5.22)	0.78 (4.90)	1.69 (4.23)	0.62 (3.79)
T_8	Phage alone	0.96 (4.98)	0.21 (4.32)	0.52 (3.72)	0.27 (3.44)
Mean		**(5.14)**	**(4.75)**	**(4.02)**	**(3.66)**
		Formulation	**Days**	**F x D**	
	S. Em±	0.033	0.023	0.066	
	CD @ 1%	0.12	0.09	0.25	

Phages @ ~10^8 PFU/ml, 30 ml per pot)
In control, no phage activity was observed
* - Figures in parenthesis are log-transformed values.

Evaluation of bacteriophages in integrated disease management

A glass house experiment was conducted on the integrated disease management of bacterial wilt disease development on both tomato (Cv. Pusa ruby) and brinjal (Cv. Manjri hybrid) including bacteriophages as components. Treatments were imposed under a completely randomized design and the observations were recorded on per cent disease severity after 25 days of inoculation. For Plant Growth-Promoting Rhizobacteria (PGPR), an effective *Pseudomonas fluorescence* strain (native isolated proved to be effective in *invitro* studies against RS6 strain of *R. solanacaeraum*) against *R. solanacearum* (nearly 10^8 CFU/mL) was used and applied to soil near root zone at 20 ml per pot wherever experimented. The remaining treatment solutions (Treatments are mentioned in Table 2) were prepared as per recommended doses. A similar kind of experiment at the same concentrations was performed on brinjal (Cv. Manjri hybrid) with the same bacterial strain used for tomato which was proved for its pathogenicity on brinjal too. Symptoms of wilting were graded from zero to five (0 - No wilting, 1 - Flaccidity of leaves, 2 - Yellowing of leaves, 3 - Yellowing of leaves and Stunting of the plant, 4 - Partial wilting, 5 - Complete wilting). The PDI is a formula used to quantify the severity of a disease as per the below formula.

PDI = (Sum of all individual disease ratings)/(Maximum disease rating × Total number of samples observed) × 100

RESULTS

A total of six *R. solanacearum* isolates (RS 1, 2, 3, 4, and 5) were purified from diseased solanaceous brinjal and tomato plants and confirmed for their pathogenicity. Four isolates (RS3, RS4, RS5 and RS6) were found pathogenic in both tomato and brinjal seedlings. Flagging of one or two leaves on plants, usually at the top portions of plants is the visual symptom, thereafter, rapidly kills plants as quickly as 4-7 days.

Water and soil samples were screened for isolation of phages against *R. solanacearum*. Fairly, nine in thirty water samples (30%) showed lysis activity on *R. solanacearum.* Soil samples collected from brinjal and tomato fields infected with bacterial wilt showed phage activity (33%) rather than uninfected fields (details not provided here). Among the isolated phages, two phages of *R. solanacearum viz.,* FRspv1

Table 2. Bacteriophage formulations on tomato bacterial wilt development

Tr. No	Treatment details	Per cent disease severity in tomato	Per cent disease reduction over control
T_1	Pregelatinized corn flour	0.00 (1.00)*	100.00
T_2	Casecrete	26.67 (3.67)	71.42
T_3	Skim milk	26.67 (3.67)	71.42
T_4	Pregelatinized corn flour + Casecrete	0.00 (1.00)	100.00
T_5	Pregelatinized corn flour+ Skim milk	0.00 (1.00)	100.00
T_6	Casecrete+ Skim milk	0.00 (1.00)	100.00
T_7	Pregelatinized corn flour + Casecrete + Skim milk	0.00 (1.00)	100.00
T_8	Phage alone (Unformulated)	0.00 (1.00)	100.00
T_9	Control	93.34 (9.35)	-
	SEm ± CD at 5%	1.26 5.14	

*- √x+1 values

and FRspv2 phages showed clear plaque morphology with plaque sizes of 2.5-4.0 and 1.0- 2.0 mm respectively named as FRspv1 (*R. solanacearum* phage variant 1) and FRspv2 were used for further studies.

Protective formulations on bacteriophage population density

The evaluation of the bacteriophage population across different treatments over time (4, 8, 12, and 16 days after storage - DAS) revealed significant variation in phage stability depending on the formulation. The population of bacteriophages is expressed in PFU/g (Plaque Forming Units per g).

On fourth day, T6 (Casecrete + Skim Milk) recorded the highest phage population at 1.84×10^5 PFU/g (log value 5.27), followed by T7 (Pregelatinized Corn Flour + Casecrete + Skim Milk) at 1.67×10^5 PFU/g (5.22).T8 (Phage alone) had the lowest phage population at 0.96×10^5 PFU/g (4.98), indicating a rapid decline in phage stability without any protective agents.

There was a two-fold reduction phage count on eighth day of the experiment in all formulated phage treatments and T4 (Pregelatinized Corn Flour + Casecrete) maintained the highest phage population at 0.80×10^5 PFU/g (4.90), while T7 (Pregelatinized Corn Flour + Casecrete + Skim Milk) followed closely with 0.78×10^5 PFU/g (4.90). T8 (Phage alone) showed a further significant reduction to 0.21×10^5 PFU/g (4.32), indicating a sharp decline in the absence of stabilizers.

Phage activity was observed in both formulated and unformulated phages at 4 $\log_{10}$ PFU/mL even after twelve and sixteen days after phage application. On the twelfth day, the population was reduced approximately by five times in all formulated phages. T7 (Pregelatinized Corn Flour + Casecrete + Skim Milk) showed the highest phage population at 1.69×10^4 PFU/g (4.23), indicating better long-term stability with the combined formulation. The lowest population was observed in T8 (Phage alone) at 0.52×10^4 PFU/g (3.72), indicating the need for protective agents to ensure phage viability over time.

On the sixteenth day, a phage count was done for all treatments. T2 (Casecrete) and T7 (Pregelatinized Corn Flour + Casecrete + Skim Milk) showed relatively better phage populations at 0.63×10^4 PFU/g (3.80) and 0.62×10^4 PFU/g (3.79) respectively. T5 (Pregelatinized Corn Flour + Skim Milk) recorded the lowest phage population at 0.29×10^4 PFU/g (3.46).

The overall analysis of the experiment for 16 days showed that phage formulations (such as Casecrete,

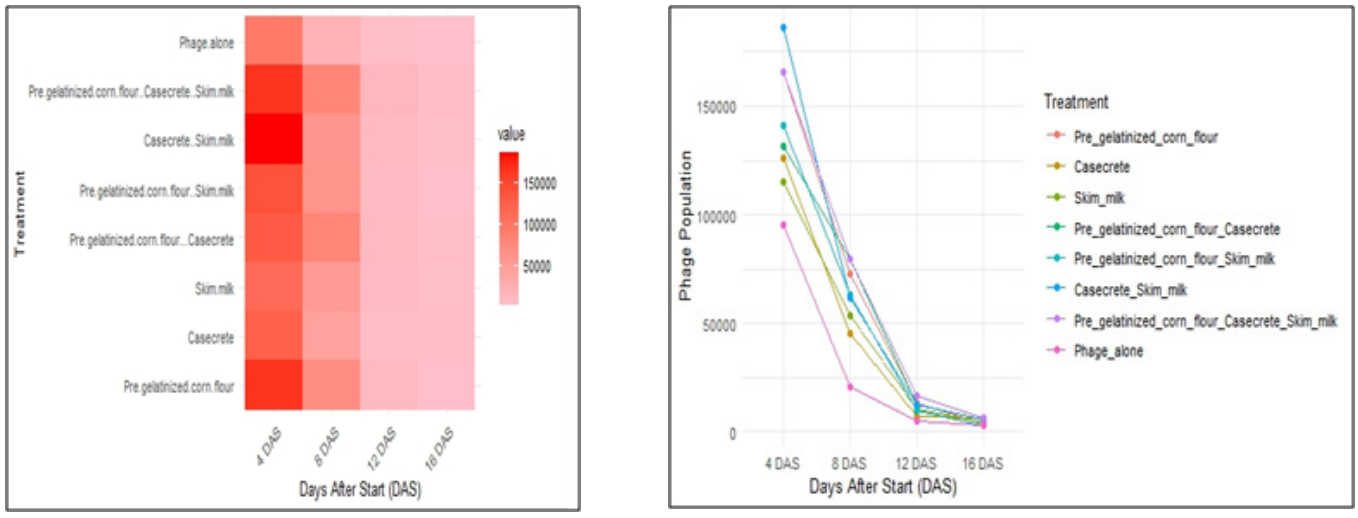


Figure 1. Phage activity in different formulations over 16 days of application.

Pregelatinized Corn Flour, and Skim Milk) generally maintained higher phage populations compared to T8 (Phage alone). The combination of Pregelatinized Corn Flour, Casecrete, and Skim Milk (T7) provided the most consistent and stable results across all time points, indicating its efficacy in preserving phage viability. Phage alone (T8) showed the lowest stability, with a significant reduction in phage population over time, emphasizing the importance of protective formulations for phage therapy in bacterial disease management (Figure 1).

Study on bacteriophage formulations in reducing bacterial wilt disease development

Bacteriophages being susceptible to various elements of the harsh environment in soil, few formulations and their combinations that were supposed to increase phage stability and viability were studied on the reduction of wilting severity over unformulated phages on tomato plants in glass house experiments for 35 days. The results showed that all the formulations showed no wilting symptoms except Casecrete protein and Skimmed Milk where 26.67% disease severity was noticed which also expressed delayed wilting symptoms (Table 2). The reason might be the survival of the phage populations in soil microflora to an extent of 4-5log PFU/mL both in unformulated as well as formulated as studied in the earlier case of phage population.

Evaluation of bacteriophages in integrated disease management

Wilting was observed in untreated control showing top most leaves wilted early by rolling leaves downwards after 10 days of treatment application in the tomato experiment. But in other treatments, wilting was not distinguishable within 25 days of observation, hence other parameters like flaccidity, yellowing and stunting were considered for recording disease severity.

Observation of Percent disease severity on the 25th day on tomatoes revealed that T4 (Phage mix alone) recorded 0.00% disease severity with 100% disease reduction, making it the most effective treatment in controlling bacterial wilt in tomatoes. T8 (COC and Streptocycline + Bleaching powder + Phage mix) also performed exceptionally well, showing 5.00% disease severity and 94.11% disease reduction over control. Treatments like T2 (Bleaching powder), T3 (COC + Streptocycline), T5 (PGPR + Phage mix), and T6 (Bleaching powder + Phage mix) all resulted in 20.00% disease severity with a significant 76.47% disease reduction compared to the control. The least effective treatment, aside from the control, was T7 (COC and Streptocycline + Phage mix), which had 30.00% disease severity and 64.70% disease reduction. T9 (Control) showed the highest disease severity at 85.00%, with no disease reduction (Table 3, Figures 2, 4A).

Similar kind of observations were made in Brinjal experiment resulting in Phage mix alone (T4) and its combinations, particularly T5 (PGPR + Phage mix), T6 (Bleaching Powder + Phage mix), and T8 (COC and Streptocycline + Bleaching powder + Phage mix), all achieved the best results in brinjal with 5.00% disease severity and a 94.44% reduction in disease. T3 (COC + Streptocycline) also performed well, reducing the disease severity to 15.00% with 83.33% disease reduction. T1 (*Pseudomonas fluorescens* - PGPR) resulted in 20.00% disease severity, achieving 77.78% disease reduction, while T2 (Bleaching powder) resulted in 35.00% disease severity and 61.11% disease reduction. T9 (Control) showed the highest disease severity at 90.00%, with no disease reduction (Table 3, Figures 3, 4B).

Our studies significantly proved that the use of phage mix, either alone or in combination with other treatments, was highly effective in both tomato and brinjal for reducing the incidence of bacterial wilt. Phage mix alone (T4) provided



Figure 2. Evaluation of bacteriophages in IDM of tomato bacterial wilt.

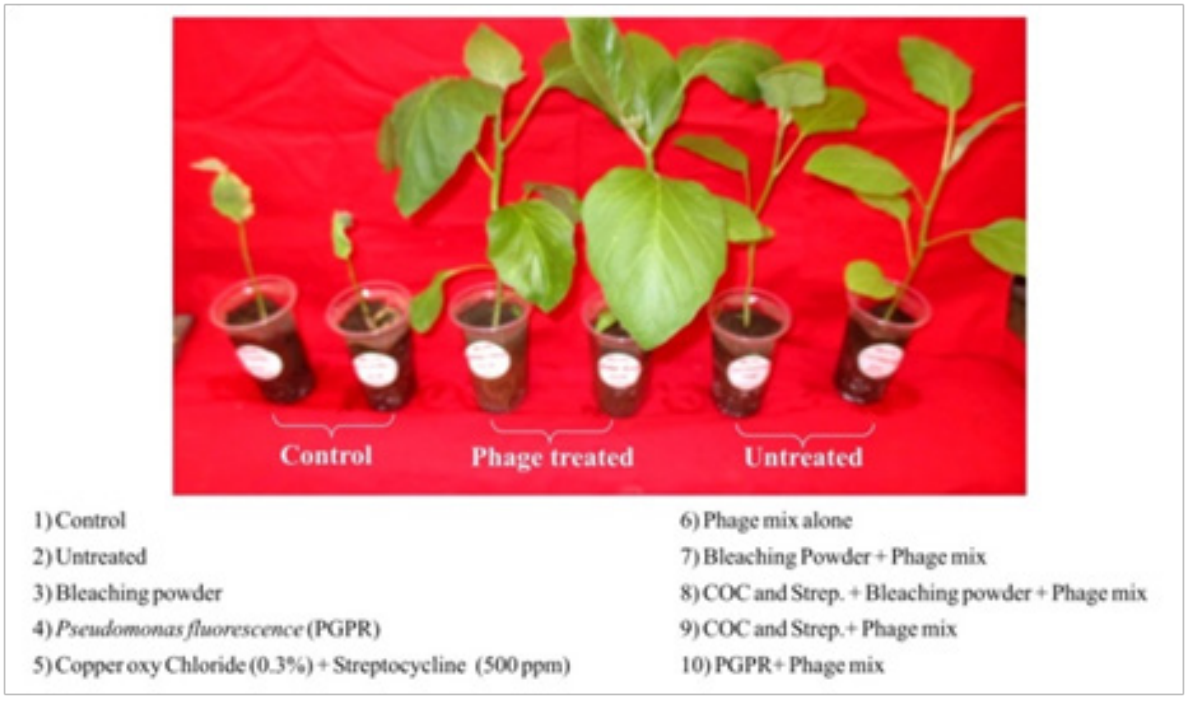


Figure 3. Evaluation of bacteriophages in IDM of Brinjal bacterial wilt.

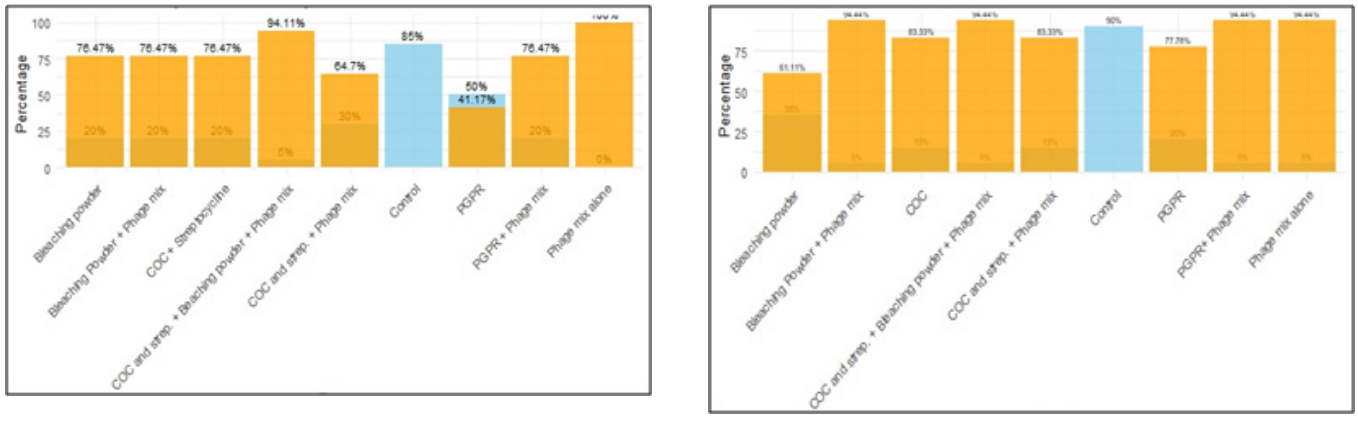


Figure 4. Graph representing bacterial wilt per cent disease severity and disease reduction in integrated disease management experiment. (A) tomato, (B) brinjal.

Table 3. Evaluation of bacteriophages in integrated disease management on tomato and brinjal bacterial wilt

Tr. No.	Treatment details	Dosage	Tomato		Brinjal	
			Per cent disease severity	Per cent Disease reduction over control *	Per cent disease severity	Per cent Disease reduction over control *
T_1	*Pseudomonas fluorescens* (PGPR)	30 ml/pot (~10^8 CFU/ml)	50.00 (6.79)*	41.17	20.00 (4.58)*	77.78
T_2	Bleaching powder	1g/pot	20.00 (4.58)	76.47	35.00 (5.28)	61.11
T_3	Copper oxy Chloride (0.3%) + Streptocycline (500 ppm) (COC)	0.3% + 500ppm	20.00 (4.58)	76.47	15.00 (3.69)	83.33
T_4	Phage mix alone	~10^8 PFU/ml (20 ml)	0.00 (1.00)	100.00	5.00 (1.90)	94.44
T_5	PGPR+ Phage mix	30 ml/pot + ~10^8 PFU/ml (20 ml)	20.00 (3.00)	76.47	5..00 (1.90)	94.44
T_6	Bleaching Powder + Phage mix	1 g/ pot + ~10^8 PFU/ml (20 ml)	20.00 (3.00)	76.47	5.00 (1.90)	94.44
T_7	COC and strep. + Phage mix	0.3% + 500ppm + ~10^8 PFU/ml (20 ml)	30.00 (4.40)	64.70	15.00 (3.69)	83.33
T_8	COC and strep. + Beaching powder + Phage mix	30 ml/pot + 0.3% + 500ppm + 0.25mM + ~10^8 PFU/ml (20 ml)	5.00 (1.90)	94.11	5.00 (1.90)	94.44
T_9	Control	-	85.00 (9.23)		90.00 (9.53)	
	S. Em± CD at 1%		1.27 3.69		0.75 2.95	

* - √x+1 value

100% disease reduction in tomatoes and 94.44% in brinjal, indicating its significant potential as a biocontrol agent. Treatments that combined phage mix with other agents, such as PGPR (T5), Bleaching powder (T6), and COC and Streptocycline (T8), also performed exceptionally well, achieving high disease reduction rates. Chemical treatments like COC and Streptocycline showed good control in both crops, but their effectiveness was enhanced when combined with phage treatments. The data indicate that phage-based treatments, especially in combination with other biological or chemical agents, are highly effective in reducing bacterial wilt severity in both tomato and brinjal. This suggests the potential for integrating bacteriophages into sustainable disease management strategies for these crops.

DISCUSSION

Phage stability is the main concern in biological management when phages are applied directly to the phyllosphere (Jones *et al.,* 2007). Studies in fields and labs have shown that sunlight, high temperatures, fluctuating pH levels, strong oxidizing environments, and flowing water can quickly render phages inactive (Ignoffo *et al.*, 1997; Iriarte *et al.*, 2012). However, for controlling bacterial wilt, applying phages to the root zone (rhizosphere) avoiding sunlight, is the most damaging environmental factor for phages, and thereby oxidative. But other challenges like entrapment of phages within bacterial biofilms (Storey & Ashbolt, 2001) or reversibly sticking to soil particles, particularly clay, low soil pH can inactivate phages. Phages can become trapped in biofilms (Storey & Ashbolt, 2001), reversibly adsorb to particles of soil, such as clay and be inactivated by low soil pH. Physical refuges can protect bacteria from coming into contact with phages.

Several materials were identified that increased residual activity of insect viruses and other microbial pesticides by protecting against sunlight UV and/or rain leaching, including antioxidants and oxidative enzymes aromatic/ heterocyclic amino acids, light adsorbing or reflecting dyes, activated charcoal, starch- and flour, alkaline gluten, casecrete, lignin-based formulations, *etc.* (McGurie *et al.,* 1990; Ignoffo & Garcia, 1992; Behle *et al.,* 2003). Among these, pregelatinized corn flour, casecrete, and skim milk can enhance the stability of bacteriophages (phages) by providing protective matrices, stabilizing agents, and moisture control. Pregelatinized corn flour can act as a moisture-retaining agent and a protective barrier around the phages, minimizing their exposure to environmental stressors like heat, UV radiation, and desiccation. Casecrete is known to encapsulate the phages, offering them protection against harsh environmental conditions (like extreme temperatures or pH shifts) that would normally degrade their viability. On the other hand, Skim milk contains proteins (like casein) and lactose, which can form a protective coating around phage particles, preventing them from inactivation due to external stresses such as drying or heat (Gašic *et al.*, 2018; Balogh, 2003 and Chandraprakash *et al.,* 2018).

Our study showed that there was continuous availability of phages in high proportions even after sixteen days. From the fourth day to the sixteenth day, approximately only twenty-fold reductions in population were observed for formulated phages. Whereas the unformulated phages significantly differed from formulated phages, they also retained high concentrations of phage over a week from 0.96 x 10^5 to 0.27 x 10^4 PFU/g.

The phages were also integrated with other components of disease management wherein pretreatment of plants with two phages alone prevented wilting after subsequent inoculation with the pathogen for both tomato and brinjal plants. Phages performed better than other individual treatments of *Pseudomonas fluorescence*, bleaching powder and Copper oxychloride and Streptocycline. Despite the challenges, these phages hold significant promise as valuable tools for reducing disease, offering a range of practical applications. From this study, it is possible to say that phage mixtures alone can be effectively used for wilting reduction in tomato and brinjal or at least, wilting was suppressed for a longer time of about 10-15 days when compared to other treatments. Fujiwara *et al.* (2011) showed that pre-treating tomato seedlings with a specific phage (FRSL1) significantly limited the ability of the bacteria to enter, grow within, and move throughout the plant's roots. Iriarte *et al.* (2012) further supported this, demonstrating that applying phages or phage mixtures to the soil around tomato plants at the same time as bacterial inoculation offered the best control. Applying phages three days before inoculation showed some effectiveness, but applying them three days after proved entirely ineffective. Similar observations were made by Fujiwara *et al.* (2011), Bhunchoth *et al.* (2015) and Kalpage and De costa (2014). These cocktails ideally contain several phages with different host specificities, replication mechanisms and/or infection cycles (Jones *et al.,* 2007). This would be the other possible reason, the bacteriophages immediately found the host and their complete invasion led to no wilting and vigorous growth. According to three consecutive field trials done by Balogh (2003) using unprocessed phages showed a disease reduction of 9-20% compared to untreated plants. Furthermore, studies by Frampton *et al.* (2012) demonstrated even greater effectiveness (12-43% disease reduction) when using a formulated phage product (Casecrete). The disease reduction observed in this current study falls well within this established range of effectiveness for phage therapy against bacterial spots in tomatoes.

CONCLUSION

The current study sheds light on the potential application of bacteriophages as biocontrol agents. It highlights that phages can serve as effective alternatives to hazardous chemicals. The initial success of bacteriophage formulations in greenhouse experiments is promising. This study demonstrates the potential of these newly discovered phages in combating solanaceous bacterial wilt. The phages effectively eliminated the *R. solanacearum* pathogen in both *in vitro* and *in vivo*, with or without integration into existing disease management strategies.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Department of Science and Technology for providing INSPIRE – Junior Research Fellowship for completion of Doctoral thesis of the corresponding author. The authors are thankful to the Department of Plant Pathology, University of Agricultural Sciences, Dharwad for providing the necessary facilities for conducting experiments.

REFERENCES

Álvarez, B., Biosca, E. G., and Lopez, M. M. 2010. On the life of *Ralstonia solanacearum*, a destructive bacterial plant pathogen. In Mendez-Vilas A (ed). Current research, technology and education topics in applied microbiology and microbial biotechnology (pp. 267-270), Badajoz, Formatex, Spain.

Bae, J. Y., Wu, J., Lee, H. J., Jo, E. J., Murugaiyan, S., Chung, E., and Lee, S-W. 2012. Biocontrol potential of a lytic bacteriophage PE204 against bacterial wilt of tomato. *J Microbiol Biotechnol*, **22**(12): 1613-1620. https://doi.org/10.4014/jmb.1208.08072 PMid:23221522

Balogh, B., Jones, J. B., Momol, M. T., Olson, S. M., and Obradovic, A. 2003. Improved efficacy of newly formulated bacteriophages for management of bacterial spot on tomato. *Plant Dis,* **87**: 949-954. https://doi.org/10.1094/PDIS.2003.87.8.949 PMid:30812801

Balogh, B, Jones, J. B., Iriarte, F. B., Momol, M. T. 2010. Phage therapy for plant disease control. *Curr Pharm Biotechnol.* **11**(1): 48-57. https://doi.org/10.2174/138920110790725302 PMID: 20214607.

Behle, R. W., Tamez-guerra, P., and Guire., M. R, 2003. Field activity and storage stability of *Anagrapha falcifera* nucleopolyhedrovirus (of MNPV) in spray-dried lignin-based formulations. *J Econ Entomol,* **96**: 1066-1075. https://doi.org/10.1093/jee/96.4.1066 PMid:14503576

Buttimer, C., McAuliffe, O., Ross, R. P., Hill, C., O'Mahony, J., and Coffey, A. 2017. Bacteriophages and bacterial plant diseases. *Front Microbiol*, **8**: Article 34. https://doi.org/10.3389/fmicb.2017.00034 PMID: 28163700; PMCID: PMC5247434.

Bhunchoth, A., Phironrit1, N., Leksomboon, C., Chatchawankanphanich, O., Kotera, S., Narulita, E., … Yamada, T. 2015. Isolation of *Ralstonia solanacearum* infecting bacteriophages from tomato fields in Chiang Mai, Thailand and their experimental use as biocontrol agents. *J Appl Microbiol*, **118**: 1023-1033. https://doi.org/10.1111/jam.12763 PMid:25619754

Carstens, A. B., Djurhuus, A. M., Kot, W., Jacobs-Sera, D., Hatfull, G. F., and Hansen, L. H. 2018. Unlocking the potential of 46 new bacteriophages for biocontrol of *Dickeya Solani*. *Viruses.* **10**(11): Article 621. https://doi.org/10.3390/v10110621 PMid:30423804 PMCid:PMC6267328

Chandraprakash, S., Manonmani, K., Revathy, N., and Dev, E. K. 2018. Management of bacterial leaf blight disease of paddy (*Oryza sativa*) caused by *Xanthomonas oryzae pv. oryzae* using bacteriophages. *Int J Curr Microbiol App Sci*, **7**: 3066-3080

Doffkay, Z., Dömötör, D., Kovács, T., and Rákhely, G. 2015. Bacteriophage therapy against plant, animal and human pathogens. *Acta Biol* Szeged, **59**: 291-302.

Frampton, R. A., Pitman, A. R. and Peter, C. F. 2012. Advances in bacteriophage-mediated control of plant pathogens. *Int J Microbiol*. p11. https://doi.org/10.1155/2012/326452

Fujiwara, A., Fujisawa, M., Hamasaki, R., Kawasaki, T., Fujie, M., and Yamada, T. 2011. Biocontrol of *Ralstonia solanacearum* by treatment with lytic bacteriophages. *Appl Environ Microbil*, **77**: 4155-4162. https://doi.org/10.1128/AEM.02847-10 PMid:21498752 PMCid:PMC3131639

Gašić, K., Kuzmanović, N., Ivanović, M., Prokić, A., Šević, M., and Obradović, A. 2018. Complete genome of the *Xanthomonas euvesicatoria* specific bacteriophage KF1, its survival and potential in control of pepper bacterial spot. *Front Microbiol*. **9**: 2021. https://doi.org/10.3389/fmicb.2018.02021 PMid:30210484 PMCid:PMC6123377

Grey, B. E., and Steck, T. R. 2001. The viable but nonculturable state of *Ralstonia solanacearum* may be involved in long-term survival and plant infection. *Appl Environ Microbiol*, **67**(9): 3866-3872. doi: 10.1128/AEM.67.9.3866-3872.2001. https://doi.org/10.1128/AEM.67.9.3866-3872.2001 PMid:11525979 PMCid:PMC93103

Hayward, A. C. 2000. *Ralstonia solanacearum*. Encyclopedia of microbiology, Vol. 4. Academic Press, Inc., San Diego, CA.

Hong, J. C., Momol, M. T., Jones, J. B., Ji, P., Olson, S. M., and Allen, C. 2008. Detection of *Ralstonia solanacearum* in irrigation ponds and aquatic weeds associated with the ponds in North Florida. *Plant Dis*. **92**(12): 1674-1682. https://doi.org/10.1094/PDIS-92-12-1674 PMid:30764299

Ignoffo, C. M., and Garcia, C. 1992. Combinations of environmental factors and simulated sunlight affecting activity of inclusion bodies of the *Heliothis* (lepidoptera : Noctuidae) nucleopolyhedrosis virus. *Environ Entomol,* **21**: 210-213. https://doi.org/10.1093/ee/21.1.210

Ignoffo, C. M., Garcia, C., Saathoff, S. G. 1997. Sunlight stability and rain-fastness of formulations of *Baculovirus heliothis*. *Environ Entomol,* **26**: 1470-1474. https://doi.org/10.1093/ee/26.6.1470

Iriarte, F. B., Obradovic, A., Wernsing, M. H., Jackson, L. E., Balogh, B., Hong, J. A., … Vallad, G. E. 2012. Soil-based systemic delivery and phyllosphere *in vivo* propagation of bacteriophages. *Bacteriophage,* **2 (4)**: 215-224. https://doi.org/10.4161/bact.23530 PMid:23532156 PMCid:PMC3594209

McGuire, R., Shasha, B. S., Lewis, L. C., and Nelsen, T. C. 1990. Residual activity of granular starch-encapsulated *Bacillus thuringiensis*. *J Econ Entomol,* **87**: 631-637. https://doi.org/10.1093/jee/87.3.631

Jain, L., Kumar, V., Jain, S. K., Kaushal, P., and Ghosh, P. K. 2023. Isolation of bacteriophages infecting *Xanthomonas oryzae* pv. *oryzae* and genomic characterization of novel phage vB_ XooS_NR08 for biocontrol of bacterial leaf blight of rice. *Front Microbiol.* **14**: Article 1084025. https://doi.org/10.3389/fmicb.2023.1084025 PMid:37007514 PMCid:PMC10061587

Jones, J. B., Jackson, L. E., Balogh, B., Obradovic, A., Iriarte, F. B., and Momol, M. T. 2007. Bacteriophages for plant disease control. *Annu Rev Phytopathol,* **45**: 245-262. https://doi.org/10.1146/annurev.phyto.45.062806.094411 PMid:17386003

Kalpage, M. D., and De Costa, D. M. 2014, Isolation of bacteriophages and determination of their efficiency in controlling *Ralstonia solanacearum* causing bacterial wilt of tomato. *Trop Agric Res,* **26**(1): 140-151. https://doi.org/10.4038/tar.v26i1.8079

Nga, N. T. T., Tran, T. N., Holtappels, D., Kim, N. N. L., Hao, N. P., Vallino, M. 2021. Phage biocontrol of bacterial leaf blight disease on welsh onion caused by *Xanthomonas axonopodispvallii*. *Antibiotics*, **10**: Article 517. https://doi.org/10.3390/antibiotics10050517 PMid:34062921 PMCid:PMC8147253

Storey, M. V., and Ashbolt, N. J. 2001. Persistence of two model enteric viruses (B408 and MS-2 bacteriophages) in water distribution pipe biofilms. *Water Sci Technol,* **43**: 133-138. https://doi.org/10.2166/wst.2001.0724

Yabuuchi, E., Kosako, V., Yano, I., Hotta, H., and Nishiuchi, Y. 1995. Transfer of two Burkholderia and an Alcaligenes species to *Ralstonia Gen*. nov.: Proposal of *Ralstonia picketii* (Ralston, Palleroni and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. *Microbiol Immunol,* **39**: 897-904. https://doi.org/10.1111/j.1348-0421.1995.tb03275.x PMid:8657018

Yuliar, N. Y. A., and Toyota, K. 2015. Recent trends in control methods for bacterial wilt diseases caused by *Ralstonia solanacearum*. *Micro Environ*, **30**(1): 1-11. https://doi.org/10.1264/jsme2.ME14144 PMid:25762345 PMCid:PMC4356456