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Scientific Article

Evaluation of the ΦXaF18 bacteriophage formulation for the control of *Xanthomonas euvesicatoria* in chili pepper plants under field conditions

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ABSTRACT

Background/Objective. *Xanthomonas euvesicatoria* (Xe) is the causal agent of bacterial spot in chili pepper crops, a disease that is commonly managed with agricultural antibiotics. As a biological alternative, bacterial viruses (bacteriophages) offer a promising approach to the control of plant pathogenic bacteria. The objective of this study was to evaluate the biological effectiveness of the bacteriophage ΦXaF18 formulated with UV radiation protection (Fagolytic) for the control of Xe in chili pepper plants under open-field conditions and in commercial field plots.

Materials and Methods. Experiments were conducted in block or completely random arrangement; five treatments were evaluated under open-field and field conditions: 1) Fagolytic, 2) Unformulated ΦXaF18 bacteriophage, 3) LS formulation with ΦXaF18 bacteriophage, 4) Kasumin® agricultural antibiotic, 5) Untreated control. The number of leaves with symptoms, number of chlorotic spots and the severity index were measured with a 6-level ordinal scale (0-5) and viral concentration in foliage. Data were statistically analyzed with an analysis of variance and a Tukey test or a nonparametric Kruskal-Wallis test ($p \leq 0.05$).

Results. Fagolytic reduced bacterial spot severity by 32% and chlorotic spots by 57% compared to unformulated bacteriophage ΦXaF18 under open-field conditions. In the field, Fagolytic significantly decreased the severity index in site B of experimental plot 1 ($p \leq 0.05$), with an efficacy comparable to the antibiotic Kasumin®. In addition, at site A, Fagolytic showed a significantly higher viral concentration of ΦXaF18 on the foliage at 48 h after the application of the treatments. Whereas at the time of application (preventive, simultaneous or combined) it had no significant effect on the severity of the disease.

Conclusion. Fagolytic proved to be an effective biological alternative for the control of bacterial spots (caused by Xe) in chili pepper plants, by reducing the severity of the disease and maintaining its persistence in the foliage, with comparable results to the Kasumin® antibiotic both under open-field conditions and in commercial plots at the field level.

Keywords: Bacterial spot, Bacteriophages, Fagolytic, Biological control

INTRODUCTION

In Mexico, chili pepper (*Capsicum annuum*) cultivation is one of the main horticultural activities, with an estimated economic value of over 17 billion pesos and an average yield of 19 t ha^{-1}. Mexico ranks second in global production, behind only China (SIAP, 2019). Moreover, Mexico is recognized as a center of origin and domestication of this species, and it is cultivated across nearly the entire country, encompassing most known varieties (Chew-Madinaveitia *et al*., 2008).

Several bacterial diseases affect chili pepper crops, with bacterial spot being one of the most significant (Vauterin *et al*., 2000; Jones *et al*., 2004; Voloudakis *et al*., 2005; Sadunishvili *et al*., 2015). This disease is mainly caused by *Xanthomonas euvesicatoria* (Xe; Jones *et al*., 2004). The most commonly used control methods include copper-based compounds and agricultural antibiotics (Balogh *et al*., 2003; Iriarte *et al*., 2012). However, the prolonged and repeated use of these strategies has led to the emergence of resistant strains (Voloudakis *et al*., 2005; Carrillo-Fasio *et al*., 2001; Balogh *et al*., 2003; Shenge *et al*., 2014).

The use of beneficial microorganisms to manage crop diseases is a safe, eco-friendly strategy that has attracted attention in the field of crop protection (Ayaz *et al*., 2023). For controlling phytopathogenic bacteria, the use of bacterial viruses, known as bacteriophages or "phages", has proven to be a viable alternative to conventional treatments (Ravensdale *et al*., 2007; Balogh *et al*., 2008; Chae *et al*., 2014; Rombouts *et al*., 2016). Plant phage therapy offers many benefits, including high specificity to the bacterial host, lack of toxicity to eukaryotic cells, the ability to infect antibiotic-resistant bacteria, and suitability for use in pesticide-free crops (Halawa, 2023). However, its application in greenhouse and field conditions may face some challenges.

When applied to plant foliage, phages are exposed to adverse climatic conditions that can compromise their integrity (Jones *et al*., 2007). Solar UV radiation is the main threat, while other factors such as desiccation, temperature fluctuations, pH, and even rainfall wash-off can rapidly reduce phage persistence on leaf surfaces (Balogh *et al*., 2003; Iriarte *et al*., 2007). Various compounds and formulations have been proposed as alternatives to protect phages from ultraviolet radiation (Balogh *et al*., 2003; Iriarte *et al*., 2007; Born *et al*., 2015; Khalil *et al*., 2016; Wdowiak *et al*., 2023). Among these, notable examples include certain plant pigments and the combination of skim milk powder and sucrose (Born *et al*., 2015; Balogh *et al*., 2003).

Nevertheless, most of these studies have been limited to laboratory conditions, and only a few evaluations have been carried out in greenhouses or fields, even though these settings are essential to validate the effectiveness of formulations under real conditions (Dy *et al*., 2018). Therefore, the aim of this study was to determine the biological effectiveness of the lytic bacteriophage ΦXaF18 in a formulation called Fagolytic, against bacterial spot caused by Xe in chili pepper crops under commercial field conditions.

MATERIALS AND METHODS

Open-field trial with partial environmental control

Plant material and Xe inoculation. Seeds of poblano chili, variety Ancho "San Luis", were sown in plastic trays using a sterile substrate composed of 37.5% sand, 37.5% soil, 12.5% peat, 3.5% humus, and 9% perlite. Transplanting was carried out into 1 L Styrofoam cups (~600 g of substrate per cup) after the plants developed two true leaves, and they were maintained under greenhouse conditions. Two identical fertilizations were applied, with a total dose of 70-70-70 (20 and 40 days after sowing) using Triple-16, and irrigation was provided every two days. After 35 days from sowing, the plants were acclimatized outdoors in the cups, where the open-field experiment was conducted.

The Xe strain BV801, from the CIATEJ Phytopathology Laboratory collection, was used for inoculation. The bacterial inoculum was prepared from an overnight culture in NYG liquid medium (0.3% yeast extract, 0.5% peptone, 2.0% glycerol), centrifuged at 10000 rpm for 15 min, and the supernatant was discarded. The pellet was resuspended in sterile distilled water, and this process was repeated twice more. The inoculum was then adjusted to a concentration of 1×10^8 CFU (colony-forming units) mL^{-1} ($OD_{600} = 1.0$). The bacterial inoculum was applied to the foliage (1 mL per leaf) using a hand sprayer, thoroughly covering both sides of all plant leaves. The plants were then kept in plastic boxes for 18 h to ensure high relative humidity.

Preparation and application of treatments. The lytic bacteriophage ΦXaF18 was used (Ríos-Sandoval *et al*., 2020). The bacteriophage was adjusted to a concentration of 1×10^8 PFU (plaque-forming units) mL^{-1}. The Fagolytic formulation was prepared according to patent MX412087 (Ibarra-Rivera *et al*., 2024). The formulation designated LS consisted of 7.5% skim milk powder and 5% sucrose (Balogh *et al*., 2003). The antibiotic Kasumin® (RSCO-FUNG-0301K-301-052-002; Arysta LifeScience) was applied at the recommended dose of 2.0 L ha^{-1}. As a control, or untreated condition, NYG medium was applied after growth of Xe, filtered through a 0.22 µm membrane, to simulate the medium in which the ΦXaF18 phage was found. Preparation of the different treatments, except for the antibiotic, was carried out 24 h before preventive application and stored at 4 °C for later use. The antibiotic was prepared immediately before application. Complete foliage coverage on both sides was ensured by applying 1 mL per leaf.

Experimental design. The experiment was conducted in a greenhouse and under open-field conditions at the Zapopan unit of CIATEJ (20.701569, -103.474393). A completely randomized design with a 5 × 3 × 2 factorial structure (30 treatments with six replications) was used, corresponding to three factors: (A) disease management, with five levels (Fagolytic, LS formulation, unformulated ΦXaF18 phage, Kasumin® antibiotic, and untreated control); (B) time of application, with three levels (preventive; simultaneous, in which Xe and the treatment were applied at the same time—"curative"; and combined: preventive and simultaneous); (C) presence of the Xe bacterial inoculum, with two levels (with and without Xe inoculation). The experimental unit was a 1 L Styrofoam cup containing a single chili pepper plant. The response variables evaluated were the number of chlorotic spots per plant, the number of symptomatic leaves, and the number of defoliated leaves. The trial was established under open-field conditions, where both the inoculum and treatments were applied. Since favorable climatic conditions for disease development did not occur during the first two weeks after treatment application, the plants were transferred to a greenhouse, where the evaluation was completed up to 30 days after Xe inoculation.

Statistical analysis. The data obtained were subjected to analysis of variance (ANOVA, $p \leq 0.05$) and Tukey's mean comparison test ($p \leq 0.05$) when the assumptions of normality and homoscedasticity were met; when these requirements were not met, a non-parametric

Kruskal–Wallis test was performed ($p \leq 0.05$). Analyses were carried out using the Statgraphics Centurion XV statistical software (http://www.statgraphics.com/).

Field trials

Experimental plot 1 in a commercial chili pepper field in Mascota, Jalisco. In November 2018, two independent experiments were carried out in a chili pepper field of Güero type Caribe (Var. Becán, Seminis®) located in the municipality of Mascota, Jalisco (20.544465, -104.806627), provided by a local farmer. Two sites (A and B) within the field were selected, each with different levels of bacterial spot severity (visible symptoms). During the experiments, the crop was at the fruiting phenological stage. In each site, a randomized complete block design was used with four treatments: (1) Fagolytic, (2) unformulated ΦXaF18 phage, (3) antibiotic (Kasumin®), and (4) untreated control (water only), with five replications per treatment. The experimental unit (EU) was a plot with 20 plants located in a 1 m long × 0.8 m wide row (10 plants on each side of the row); the useful plot, where the response variables were measured, consisted of the two central plants within the EU. Phage treatments were applied at a concentration of 1×10^{8} PFU mL^{-1}, and the antibiotic at the recommended dose of 2.0 L ha^{-1}. Applications were carried out with a 15 L hand-held backpack sprayer equipped with a hollow-cone nozzle, applying approximately 35 mL per plant for each treatment.

Evaluated response variables. Data collection was carried out before and after treatment application (22 days after application). An ordinal severity scale was used to assess bacterial spot severity in the plants, recording disease symptoms on a scale from 0 to 5 (Table 1). The total number of leaves per plant and the number of symptomatic leaves were counted. To quantify the concentration of the ΦXaF18 phage on foliage, 10 leaves per treatment were randomly selected 48 h after treatment application. Quantitative data were analyzed using analysis of variance and Tukey's test. For qualitative data, the non-parametric Kruskal–Wallis test was applied; all analyses were conducted at a 5% significance level ($p \leq 0.05$). Statistical analyses were performed using the Statgraphics Centurion XV software (http://www.statgraphics.com/).

Table 1. Description of the ordinal severity scale used to evaluate bacterial spot (*X. euvesicatoria*) in chili pepper plants in commercial field plots located in Mascota, Jalisco.

Qualitative value	Description of the severity of bacterial spot
0	Healthy plant: absence of symptoms.
1	Plant with mild symptoms: up to 10 leaves with lesions.
2	Plant with obvious symptoms: presence of up to 20 leaves with lesions.
3	Plant with advanced symptoms: onset of defoliation and most leaves with coalescing lesions.
4	Plant with severe symptoms: 50% defoliation and lesions on all leaves.
5	Plant with terminal symptoms: 75% affected by advanced defoliation.

Determination of ΦXaF18 bacteriophage concentration on foliage. The 10 leaves collected from the experimental plot were stored in plastic bags and kept refrigerated at 8 °C until processing in the laboratory. Then, 100 mL of sterile distilled water were added to each bag and shaken continuously at 200 rpm for 20 min. Afterward, 1 mL of the sample was transferred to 1.5 mL tubes, and 400 µL of chloroform were added. The mixture was shaken for 20 min (200 rpm) and centrifuged at 13000 rpm for 10 min. Finally, the chloroform was removed by evaporation, and the presence of ΦXaF18 bacteriophages was determined using the double-layer agar technique (Kropinski *et al.*, 2009).

Experimental plot 2 in a commercial chili pepper field in Mascota, Jalisco. In November 2018, a second chili pepper field of Güero type Caribe (Var. Becán, Seminis®) located in Mascota, Jalisco (20.576549, -104.867441) was used. The same treatments, concentrations, application method, and number of replications per treatment described for experimental plot 1 were applied. The experimental unit (EU) was similar to that designed for experimental plot 1. In this trial, the only response variable evaluated was disease severity using the scale shown in Table 1; therefore, the statistical analysis of the data from this experimental plot was performed using the non-parametric Kruskal–Wallis test ($p \leq 0.05$).

RESULTS

Evaluation of the effectiveness of ΦXaF18 bacteriophages in controlling bacterial spot in chili pepper. During the trial conducted at the Zapopan unit of CIATEJ, disease severity was low, which limited the appearance of advanced symptoms such as coalescing necrotic lesions or defoliation. Regarding the number of symptomatic leaves, numerical differences were observed among treatments, with Fagolytic showing the lowest average number of affected leaves. However, statistically significant differences ($p \leq 0.05$) were found when comparing Fagolytic with the unformulated ΦXaF18 phage treatment for both the number of symptomatic leaves and chlorotic spots (Table 2). Similarly, the Fagolytic and LS formulation treatments showed a lower degree of severity, evidenced by a lower average number of chlorotic spots, with significant differences ($p \leq 0.05$) compared to the unformulated ΦXaF18 phage treatment and the untreated control. In contrast, the antibiotic Kasumin® showed no significant differences from the control treatment in either variable (number of chlorotic spots or symptomatic leaves).

Table 2. Effect of biological treatments for the management of bacterial spot in chili pepper plants under open-field/greenhouse conditions.

Treatment	**Symptoms of bacterial spot on chili pepper plants**	
	Number of leaves with symptoms	Number of chlorotic spots
Fagolytic	1.75 a	4.52 a
LS formulation	2.36 ab	5.80 a
Kasumin® antibiotic	2.41 ab	8.55 ab
Unformulated ΦXaF18 bacteriophage	2.97 b	10.55 b
No treatment	2.58 ab	11.13 b

Different letters indicate significant differences according to Tukey's test ($p \leq 0.05$).

Finally, no significant differences were observed among the different application times (preventive, simultaneous, or combined), indicating that this factor did not significantly influence symptom development (Table 3). However, as shown in Figure 1, significant differences ($p \leq 0.05$) were detected among the treatments evaluated for the variable of chlorotic spots. In this regard, the Fagolytic formulation demonstrated consistent biological effectiveness regardless of the application time, showing severity levels statistically similar ($p \leq 0.05$) to the untreated control without bacterial inoculation (Xe). In contrast, the Kasumin® treatment showed significant differences compared to the control, indicating lower relative effectiveness in disease management. Overall, phage therapy treatments showed a favorable trend in reducing the incidence of chlorotic spots.

Table 3. Effect of application timing of treatments for the management of bacterial spot in chili pepper plants under open-field conditions.

Treatment	Symptoms of bacterial spot on chili pepper plants	
	Number of leaves with symptoms	Number of chlorotic spots
Preventive application	2.28 a	7.43 a
Simultaneous application	2.30 a	7.16 a
Preventive and simultaneous application	2.66 a	9.85 a

Different letters indicate significant differences according to Tukey's test (p ≤ 0.05).

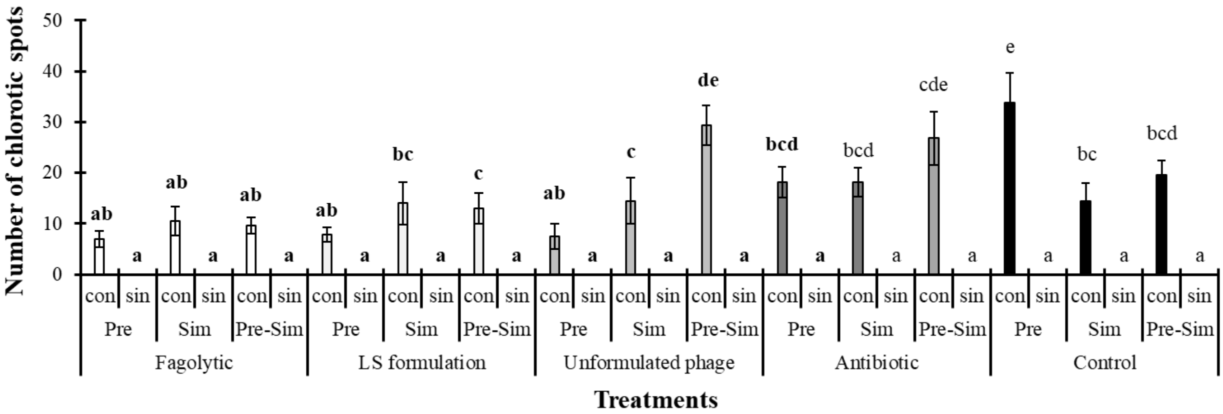

Figure 1. Effect of different treatments on the management of bacterial spot in chili pepper plants under greenhouse and open-field conditions. Different letters indicate significant differences according to Tukey's test (p ≤ 0.05). Bars in each rectangle represent ± standard error. With Xe inoculum (con), without Xe inoculum (sin). Pre: Preventive. Sim: Simultaneous. Pre-Sim: Preventive and Simultaneous.

Evaluation of the effectiveness of bacteriophages for controlling bacterial spot in experimental plot 1 in Mascota, Jalisco. In the ordinal severity scale corresponding to the initial data collection, different severity levels were observed between the two experiments (Table 4). At site A, initial disease pressure was higher compared to site B, which was reflected in more advanced symptoms, such as coalescing necrotic lesions and a greater degree of defoliation in all treatments. This initial severity level may have influenced the absence of significant differences in the final severity index evaluation (Table 4). Nevertheless, a numerical trend toward fewer symptomatic leaves was observed in the Fagolytic treatment compared to the untreated control (Table 5). At site B, the antibiotic treatment showed effectiveness comparable to that of Fagolytic, which significantly reduced the severity index compared to the control (p ≤ 0.05; Table 4), an effect clearly illustrated in Figure 2. Likewise, the number of symptomatic leaves was lower in the Fagolytic treatment compared to the control, although without reaching statistical significance (Table 5).

Table 4. Evaluation of bacterial spot severity in chili pepper plants at 0 and 22 days after treatment application for disease control in commercial experimental plot 1 located in Mascota, Jalisco.

Treatment	Start of the experiment (0 days)	22 days
	Site A	
Fagolytic	1.5 a	2.5 a
Kasumin® antibiotic	1.5 a	2.0 a
Unformulated ΦXaF18 bacteriophage	1.0 a	2.0 a
No treatment	1.0 a	2.0 a
	Site B	
Fagolytic	1.0 a	2.0 a
Kasumin® antibiotic	1.0 a	2.0 a
Unformulated ΦXaF18 bacteriophage	1.0 a	3.0 ab
No treatment	1.0 a	3.0 b

Different letters indicate significant differences according to the Kruskal–Wallis test ($p \leq 0.05$) and 95% confidence intervals of the median.

Table 5. Effect of treatments for the biological management of bacterial spot in chili pepper plants in commercial experimental plot 1 located in Mascota, Jalisco.

Treatment	Site A		Sitio B	
	Leaves with symptoms	Total leaves	Leaves with symptoms	Total leaves
Fagolytic	54.70 a	172.95 a	55.75 a	196.60 a
Kasumin® antibiotic	52.30 a	165.35 a	53.10 a	187.35 a
Unformulated ΦXaF18 bacteriophage	56.80 a	162.35 a	59.30 a	208.40 a
No treatment	65.95 a	179.85 a	76.90 a	193.50 a

Different letters indicate significant differences according to Tukey's test ($p \leq 0.05$).

Regarding the foliar persistence of the ΦXaF18 bacteriophage, significant differences in residual concentration were observed at site A between the Fagolytic treatment and the unformulated phage ($p \leq 0.05$; Table 6). Fagolytic maintained higher concentrations on foliage up to 48 h after application. In contrast, at site B, although numerical differences were recorded between the two treatments, these were not statistically significant (Table 6).

Table 6. Determination of viral concentration on chili pepper foliage at sites A and B of commercial experimental plot 1, located in Mascota, Jalisco, 48 h after application of the ΦXaF18 bacteriophage treatment.

Treatment	Concentration of bacteriophage ΦXaF18 (10^6 PFU/g of leaf tissue)	
	Site A	Site B
Fagolytic	0.84 a	0.62 a
Unformulated ΦXaF18 bacteriophage	0.23 b	0.32 a

Different letters indicate significant differences according to Tukey's test ($p \leq 0.05$).

Figure 2. Visual comparison of bacterial spot severity in chili pepper plants treated with Fagolytic (**A and C**) and untreated control (**B and D**) at 22 days after the start of the experiment in commercial experimental plot 1 located in Mascota, Jalisco.

Evaluation of the effectiveness of bacteriophages for controlling bacterial spot in experimental plot 2 in Mascota, Jalisco. The plants in this plot showed low severity indices at the beginning of the experiment across all treatments. During the evaluation period, disease development remained at low severity levels with minimal defoliation. As a result, no significant differences were observed among treatments (Table 7). In contrast, the antibiotic treatment showed the highest severity values, with significant differences ($p \leq 0.05$) compared to the bacteriophage treatments. The Fagolytic treatment did not differ significantly from the unformulated ΦXaF18 phage, although both showed a reduction in final severity compared to the beginning of the trial.

Table 7. Evaluation of bacterial spot severity in chili pepper plants at 0 and 22 days after treatment application in commercial experimental plot 2 located in Mascota, Jalisco.

Treatment	Bacterial spot disease severity index in chili pepper plants	
	Start of the experiment (0 days)	22 days
Fagolytic	1.5 a	1.0 a
Kasumin® antibiotic	1.0 a	2.0 b
Unformulated ΦXaF18 bacteriophage	1.0 a	1.0 a
No treatment	1.0 a	1.0 ab

Different letters indicate significant differences according to Tukey's test ($p \leq 0.05$).

DISCUSSION

Several bioassays have shown that the application of bacteriophages in agriculture can be effective for controlling bacterial diseases, with the goal of gradually reducing the use of antibiotics and positioning phages as an ecological alternative. However, one of the main limitations for their implementation in the field is the rapid inactivation of viral particles on foliage, mainly due to exposure to solar UV radiation, which compromises their effectiveness.

In this study, the Fagolytic formulation significantly increased the effectiveness of biological control of bacterial spot in chili pepper plants under open-field conditions. Compared to the unformulated ΦXaF18 bacteriophage, Fagolytic reduced disease severity by 32% and the number of chlorotic spots by 57%. In addition, Fagolytic showed greater persistence on foliage up to 48 h after application under field conditions. This result is relevant, since several studies have reported that unprotected bacteriophages lose their lytic activity within a few hours due to UV radiation, reducing their effectiveness to less than 24 h (Jones *et al*., 2007; Svircev *et al*., 2010; Iriarte *et al*., 2007; Balogh *et al*., 2003). This persistence represents an important advantage for sustained disease control and may improve the protection window against new infection events.

Regarding the timing of treatment application, no significant differences were observed between preventive application (24 h before inoculation), simultaneous application, or the combination of both. This suggests that the preventive strategy alone may be sufficient to achieve effective disease control under the evaluated conditions. Previous studies have indicated that preventive application can promote initial phage coverage of foliage, allowing their establishment before the arrival of the phytopathogen (Iriarte *et al*., 2012; Álvarez *et al*., 2019).

Under commercial field conditions, Fagolytic showed efficacy comparable to the antibiotic Kasumin® in reducing symptoms, particularly at site B of commercial experimental plot 1 located in Mascota, Jalisco. These results are consistent with a previous study on the control of bacterial spot in tomato (Xe), where foliar applications of formulated phages achieved efficacy levels comparable to those obtained with conventional copper- or antibiotic-based treatments (Obradovic *et al*., 2004).

In Mexico, kasugamycin, the active ingredient of Kasumin®, is widely used for the control of bacterial spot in chili pepper and tomato (Vallad *et al*., 2010). However, rapid resistance development has been reported in populations of *X. perforans* (Vallad *et al*., 2010). Another major concern is the presence of antibiotic residues in fruits, which have been linked to human health risks. In countries such as the United States, tolerance limits have been established to regulate its presence in certain fruits (US EPA, 2018).

On the other hand, the use of bacteriophages is safe for human health and does not cause phytotoxicity (Gasic *et al*., 2018). Regarding the likelihood of resistance development, it can be avoided by applying concentrations higher than 10^8 PFU mL^{-1} and/or by using mixtures or “cocktails” of different bacteriophages with the same bacterial host range (Balogh *et al*., 2010; Sadunishvili *et al*., 2015; Wei *et al*., 2017; Holtappels *et al*., 2021). Other strategies can also be employed to prevent resistance of the target bacterium to a specific phage, such as continuous monitoring (isolation) of bacterial strains treated with phages to assess subtle changes in phage susceptibility (Balogh *et al*., 2010; Holtappels *et al*., 2021).

In crop production, the application method for nearly any foliar-targeted product is spraying, and proper implementation is essential as it ensures broad coverage and adequate distribution. The Fagolytic formulation enabled efficient application through manual spraying with backpack sprayers, commonly used by farmers in the Mascota, Jalisco region, without technical difficulties.

CONCLUSIONS

The ΦXaF18 bacteriophage, formulated as Fagolytic, showed the lowest bacterial spot severity under open-field conditions, with averages of 1.8 symptomatic leaves and 4.5 chlorotic spots, significantly lower than the unformulated ΦXaF18 phage treatment (3 and 10.6, respectively). In the field, Fagolytic significantly reduced severity at site B of commercial experimental plot 1 ($p \leq 0.05$) and maintained higher residual viral concentrations up to 48 h after application. In experimental plot 2, phage treatments showed lower severity compared to the antibiotic, although no statistical differences were observed between Fagolytic and the other viral treatments. Overall, these results confirm the potential of Fagolytic as a biological alternative for managing *Xanthomonas euvesicatoria* in chili pepper plants.

Conflict of interest

The authors declare no conflict of interest.

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Author contributions

Original idea, E.E.Q.A., G.R.E.; methodology, G.I.R., E.E.Q.A., G.A.S.S., G.R.E., E.G.M.; sampling and data analysis, G.I.R., E.E.Q.A., G.A.S.S., G.R.E.; initial writing and editing, G.I.R., E.E.Q.A., G.A.S.S.; supervision, G.R.E., E.E.Q.A.; final writing and editing, G.I.R., E.E.Q.A., G.R.E., E.G.M.; funding acquisition, G.R.E., E.E.Q.A. All authors have read and approved the published version of the manuscript.

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